

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : C12Q 1/68, G01N 33/53		A1	(11) International Publication Number: WO 90/02204 (43) International Publication Date: 8 March 1990 (08.03.90)
(21) International Application Number: PCT/US89/03582		(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent).	
(22) International Filing Date: 18 August 1989 (18.08.89)			
(30) Priority data: 239,491 31 August 1988 (31.08.88) US		Published <i>With international search report.</i>	
(71) Applicant: RESEARCH DEVELOPMENT CORPORATION [US/US]; 402 North Division Street, Carson City, NV 89703 (US).			
(72) Inventors: EVINGER-HODGES, Mary, Jean ; 1030 Margate, Pearland, TX 77584 (US). BRESSER, Joel ; 2830 South Bartell, Building 3, Apartment 34, Houston, TX 77054 (US).			
(74) Agent: GOODMAN, Rosanne; Fulbright & Jaworski, 1301 McKinney Street, Houston, TX 77010 (US).			
(54) Title: MANUAL <i>IN SITU</i> HYBRIDIZATION ASSAY			
(57) Abstract			
A rapid, sensitive <i>in situ</i> hybridization assay is provided which will detect as few as 1-5 copies of target biopolymer per cell and may be accomplished in 2-4 hours. There is provided a quantitative assay which may be used to diagnose and monitor treatment of diseases.			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Fasso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic	KR	Republic of Korea	SN	Senegal
CG	Congo	LJ	Liechtenstein	SU	Soviet Union
CH	Switzerland	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
DE	Germany, Federal Republic of	MC	Monaco	US	United States of America
DK	Denmark				

1

5

-1-

10

MANUAL IN SITU HYBRIDIZATION ASSAY

15

BACKGROUND OF THE INVENTION

20

1. Field of the invention.

The present invention relates to the field of in situ hybridization assays useful for detecting as few as 1-5 copies of target nucleic acid per cell. This assay method significantly increases the sensitivity of detection of nucleic acids over other known methods. In addition, this hybridization method is accomplished with far greater speed than has been reported for other in situ assays. This present invention also provides a method for the rapid and sensitive detection of nuclear acids and proteins in the same cell.

25

2. Description of the prior art.

30

In situ hybridization provides a technique for the determination and quantitation of biopolymers such as nucleic acids (DNA and RNA) and proteins in tissues at the single cell level. Such hybridization techniques can detect the presence or absence of specific genes in tissues at the single cell level. In situ hybridization procedures may also be utilized to detect the expression

35

1 of gene products at the single cell level.

5 By the use of specific nucleic acid (RNA or DNA) probes, genetic markers for infection and other disease states may be detected. Certain genetic diseases are characterized by the presence of genes which are not present in normal tissue. Other diseased conditions are characterized by the expression of RNAs or RNA translation products (i.e. peptides or proteins) which are not expressed in normal cells. Some disease states are characterized by the absence of certain genes or gene portions, or the absence or alteration of expression of gene products or proteins.

10 Current methods allow the detection of these markers but are relatively time consuming and of limited sensitivity. Hybridization techniques are based on the ability of single stranded DNA or RNA to pair (or hybridize) with a complementary nucleic acid strand. This hybridization reaction allows the development of specific probes that can identify the presence of specific genes (DNA), or polynucleotide sequences or the transcription 15 and expression of those genes (mRNA).

20 Solution hybridization methods which require the destruction of the cell and the isolation of the nucleic acids from the cell prior to carrying out the hybridization reaction sacrifice the cellular integrity, spatial resolution and sensitivity of detection. In situ hybridization allows the detection of RNA or DNA sequences 25 within individual cells. In situ hybridization yields greater sensitivity than solution hybridization by means of eliminating the dilution of a particular target gene, nucleic acid, or protein by the surrounding and extraneous RNA and DNA of other cells. In situ hybridization also allows for the simultaneous detection of multiple substances, i.e. genes, nucleic acids or proteins within 30 individual cells, permitting the identification of a

1 particular cell expressing a cellular gene or viral
sequence. In addition, since in situ hybridization
analysis is performed and quantitated for single cells,
minimal sample and reagents are required.

5 Prior to the present invention, in situ
hybridization procedures were only capable of detecting
nucleic acids present at greater than ten copies per
cell. Such procedures required at least 8 hrs. to over 14
days to perform. Prior in situ procedures were neither
10 quantitative nor capable of performing multiple
simultaneous detections.

SUMMARY OF THE INVENTION

15 It is an object of the present invention to
provide an in situ hybridization procedure capable of
detecting polynucleotides when present at a concentration
as low as 1-5 copies per cell.

20 It is a further object of the present invention
to provide an in situ hybridization procedure capable of
detecting more than one target molecule in an individual
cell.

25 It is a further object of the present invention
to provide an in situ hybridization procedure that could
be carried out within about two to four hours.

30 It is a further object of the present invention
to provide an in situ hybridization procedure that could
be quantitative for as few as 1-5 molecules of target
nucleic acid per cell.

35 It is a further object of the present invention
to provide an in situ hybridization procedure that could
simultaneously detect multiple biopolymers.

The present invention provides a method for the
detection of biopolymers within individual cells or tissue
sections deposited on a solid support. Optimization of
each step of the procedure as provided by the present

invention allows a rapid, sensitive hybridization assay. Target biopolymer molecules may be quantitated at a level of as few as 1-5 molecules per cell. This hybridization assay may be used to detect levels of polynucleotides in cells such as bone marrow and peripheral blood, in tissue sections or in tissue cultured cells. The hybridization procedure of the present invention can detect polynucleotides in single cells with the sensitivity of as few as 1-5 molecules per cell in as little as 2-4 hours. This procedure also allows for the simultaneous detection of more than one different polynucleotide sequence in an individual cell. The present invention also allows detection of proteins and polynucleotides in the same cell.

Briefly, cells, either as single cell suspensions or as tissue slices were deposited on solid supports such as glass slides. The cells are fixed by choosing a fixative which provides the best spatial resolution of the cells and the optimal hybridization efficiency. After fixation, the support bound cells may be dehydrated and stored at room temperature or the hybridization procedure may be carried out immediately.

The hybridization step is then carried out in a solution containing a chaotropic agent such as 50% formamide, a hybrid stabilizing agent such as five times concentrated SSC solution ($1x = 0.15M$ sodium chloride and $0.015M$ sodium citrate), a buffer such as 0.1M sodium phosphate (pH 7.4), about 100 micrograms (ug)/milliliter (ml) low molecular weight DNA to diminish non-specific binding, 0.1% Triton X-100 to facilitate probe entry into the cells and about 10-20 mM vanadyl ribonucleoside complexes.

To the hybridization solution is added a probe, to hybridize with a target polynucleotide. The most preferable probe is a single-stranded RNA probe,

1 approximately 75 to 150 bases in length. An antibody
probe may be utilized to bind to a target protein or
antigen. The hybridization solution containing the probe
is added in an amount sufficient to cover the cells. The
5 cells are then incubated at 55°C for at least 30 minutes.
The probe is added at a high concentration of at least
about 1 ug/ml of hybrid mix in order to give optimal
results in this time frame.

10 The probes may be detectably labeled prior to
addition to the hybridization solution. Alternatively, a
detectable label may be selected which binds to the
hybridization product. Probes may be labeled with any
detectable group for use in practicing the invention.
Such detectable group can be any material having a
15 detectable physical or chemical property. Such detectable
labels have been well-developed in the field of
immunoassays and in general most any label useful in such
methods can be applied to the present invention.
Particularly useful are enzymatically active groups, such
20 as enzymes (see Clin. Chem., 22:1243 (1976)), enzyme
substrates (see British Pat. Spec. 1,548,741), coenzymes
(see U.S. Patents Nos. 4,230,797 and 4,238,565) and enzyme
inhibitors (see U.S. Patent No. 4,134,792); fluorescers
(see Clin. Chem., 25:353 (1979); chromophores; luminescers
25 such as chemiluminescers and bioluminescers (see Clin.
Chem., 25:512 (1979)); specifically bindable ligands;
proximal interacting pairs; and radioisotopes such as
³H, ³⁵S, ³²P, ¹²⁵I and ¹⁴C.

30 The invention of the present application which
provides optimal fixatives allowing probe entry and
blocking of non-specific probe binding and formamide
hybridization at high temperatures (55°C) provides a
hybridization assay with rapid kinetics of hybrid
formation and sensitivity of as few as 1-5 molecules per
cell.

1 The superior results of the invention of the
present application is postulated to occur by preventing
precipitation of cellular constituents onto mRNA or the
covalent modification of mRNA, the destabilization of
5 ribosomal RNA subunit binding, and promotion of
accessibility of full length mRNA for hybrid formation by
inducing single-strandedness in cellular RNA and/or DNA.
The present invention arose out of the applicant's
discovery of the strong correlation between cellular RNA
10 single-strandedness and the rapid kinetics of
hybridization which yielded a highly sensitive assay
procedure.

15 In one aspect, the present invention provides a
simple method to determine the optimal fixation/
prehybridization/hybridization/detection conditions for
any tissue type so that: (1) single molecules may be
detected, (2) cellular morphology will be preserved and
(3) the total reaction time will be reduced to 2-4 hours.

20 Briefly, in order to predict the optimal
conditions to achieve this rapid and sensitive
hybridization, a cellular specimens in multiple samples,
either in suspension or deposited on glass slides, are
exposed first to a fixative and subsequently to a
hybridization solution.

25 The fixative is selected from the group
consisting of 95% ethanol/5% acetic acid, 75% ethanol/20%
acetic acid, 50% methanol/50% acetone and 10%
formaldehyde/90% methanol (all v/v). Other useful
fixatives will be obvious to one skilled in the art as
30 long as the fixative selected allows at least a 70% shift
of double stranded to single stranded cellular
polynucleotides while maintaining cellular spatial
relationships. The duration of exposure to the fixative
is from 1 to 180 min. Preferably, 1 to 30 min., and most
35 preferably 20 min. The temperature of the fixation

1 procedure is preferably -20 to 50°C. and most preferably
2 20°C. A subsequent exposure to 70% ethanol/30% water for
3 0.5 min. to 20 min. at -20 to 30°C. may be utilized if
4 samples are to be stored prior to hybridization.

5 The hybridization solution consists of a
6 chaotropic denaturing agent, a buffer, a pore forming
7 agent, a hybrid stabilizing agent, non-specific
8 nucleotides, and a target specific probe.

9 The chaotropic denaturing agent (Robinson, D. W.
10 and Grant, M. E. (1966) J. Biol. Chem. 241: 4030;
11 Hamaguchi, K. and Geiduscheck, E. P. (1962) J. Am. Chem.
12 Soc. 84: 1329) is selected from the group consisting of
13 formamide, urea, thiocyanate, guanidine, trichloroacetate,
14 tetramethylamine, perchlorate, and sodium iodide. Any
15 buffer which maintains pH at least between 7.0 and 8.0 may
be utilized.

16 The pore forming agent is for instance, a
17 detergent such as Brij 35, Brij 58, sodium dodecyl
18 sulfate, CHAPSTM Triton X-100. Depending on the
19 location of the target biopolymer, the pore-forming agent
20 is chosen to facilitate probe entry through plasma, or
21 nuclear membranes or cellular compartmental structures.
22 For instance, 0.05% Brij 35 or 0.1% Triton X-100 will
23 permit probe entry through the plasma membrane but not the
24 nuclear membrane. Alternatively, sodium desoxycholate
25 will allow probes to traverse the nuclear membrane. Thus,
26 in order to restrict hybridization to the cytoplasmic
27 biopolymer targets, nuclear membrane pore-forming agents
28 are avoided. Such selective subcellular localization
29 contributes to the specificity and sensitivity of the
30 assay by eliminating probe hybridization to complimentary
31 nuclear sequences when the target biopolymer is located in
32 the cytoplasm. Agents other than detergents such as
33 fixatives may serve this function. Furthermore, a
34 biopolymer probe may also be selected such that its size
35

1 is sufficiently small to traverse the plasma membrane of a
cell but is too large to pass through the nuclear membrane.

5 Hybrid stabilizing agents such as salts of mono-
and di-valent cations are included in the hybridization
solution to promote formation of hydrogen bonds between
complimentary sequences of the probe and its target
biopolymer. Preferably sodium chloride at a concentration
from .15M to 1M is used; most preferably, the
concentration of sodium chloride is 0.6M.

10 In order to prevent non-specific binding of
nucleic acid probes, nucleic acids unrelated to the target
biopolymers are added to the hybridization solution at a
concentration of 100 fold the concentration of the probe.

15 Specimens are removed after each of the above
steps and analyzed by observation of cellular morphology
as compared to fresh, untreated cells using a phase
contrast microscope. The condition determined to maintain
the cellular morphology and the spatial resolution of the
various subcellular structures as close as possible to the
fresh untreated cells is chosen as optimal for each step.

20 In addition, cellular nucleic acids were stained
with about 50 ug/ml propidium iodide dye. This dye has a
specific characteristic fluorescent emission (about 480
nm, green) when the nucleic acid is single-stranded and
emits at a different wave length (about 615 nm, red) when
25 the nucleic acid is double-stranded. The dye utilized may
be dependent upon whether the target sequence for the
particular assay is RNA or DNA. If the assay is to detect
low copy numbers of DNA, then a DNA detecting dye such as
acridine orange, tetrahydrofuran, methyl green, pyronin Y
30 and azure B are used, and the nuclear DNA is analyzed for
the amount of single or double-strandedness. If instead,
the assay is to be used to detect low copy numbers of RNA,
then RNA dye such as Acridines, Azines, Xanthenes,
35 Oxazines, and Thiazines are used and the cytoplasmic RNA

1 is analyzed for the amount of single or
double-strandedness. Regardless of whether the assay is
used to analyze RNA or DNA, the optimal conditions are
reached when the nucleic acid to be detected has undergone
5 a 70% shift from double-strandedness to
single-strandedness. When the shift of the secondary
structure of the nucleic acid from double-strandedness to
single-strandedness has reached at least 70%, and there is
no decrease in the total amount of fluorescence, then the
10 conditions have been adjusted according to the present
invention and will permit optimal hybridization and
detection of as few as 1-5 molecules of target nucleic
acid within a single cell. Furthermore, the time required
for optimal hybridization can be determined from the
15 amount of time necessary for at least 70% of the cellular
nucleic acid to become single-stranded.

20 In the most preferred embodiment, the
hybridization assay of the present invention provides a
method for assaying biopolymers in a cell sample having
substantially intact membranes comprising the steps of
25 1) depositing the target cells onto a solid support,
2) fixing the cells, 3) incubating the cells with a
hybridization solution containing a single-stranded RNA
probe, 4) detecting the amount of probe hybridized to the
target nucleic acid. The samples are then washed and the
amount of target nucleic acids are determined by
quantitation either photographically through a microscope
with fluorescent capabilities or by direct reading of the
fluorescence with a Meridian ACAS 470 work station
30 (Meridian Instruments, Okemos, Michigan).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 demonstrates the optimal temperature of
the In Situ Hybridization.

Figure 2 demonstrates the kinetics of the In Situ

1 Hybridization reaction.

Figure 3 demonstrates the changes in secondary structure of cellular RNA as a function of efficiency of the In Situ Hybridization reaction.

5 Figure 4 demonstrates the sensitivity of the In Situ Hybridization reaction using a control cell line, Balb/c3T3.

10 Figure 5 demonstrates the detection of oncogenes in normal peripheral blood, normal bone marrow and chronic myelogenous leukemia (CML) by In Situ Hybridization.

Figure 6 demonstrates the detection of oncogenes in solid tissue samples by In Situ Hybridization.

15 Figure 7 demonstrates the specificity of detection of Human Immunodeficiency Virus (HIV) in positive and negative controls by In Situ Hybridization.

Figure 8 demonstrates the detection of HIV in patients with Kaposis Sarcoma (KS) or AIDS Related Complex (ARC) by In Situ Hybridization.

20 Figure 9 demonstrates the detection of HIV in patients with Acquired Immune Deficiency Syndrome (AIDS) or Lymphoma by In Situ Hybridization.

Figure 10 demonstrates the detection of HIV in seropositive (Ab+), asymptomatic, high risk individuals by In Situ Hybridization.

25 Figure 11 demonstrates the simultaneous detection by In Situ Hybridization of three oncogenes within the same peripheral blood cells of a patient with chronic myelogenous leukemia (CML). Fluorescent and enzymatic In Situ Hybridization detections are used for the analysis.

30 Figure 12 demonstrates the simultaneous detection of three oncogenes within the same peripheral blood cells of a patient with chronic myelogenous leukemia (CML). Fluorescent and colloidal gold In Situ Hybridization detections are used for the analysis.

35 Figure 13 demonstrates the simultaneous detection

1 of antigens and nucleic acids within the same cells using
In Situ Hybridization.

Figure 14 demonstrates a quantitative analysis
of In Situ Hybridization data.

5 Figure 15 demonstrates the detection of
Cytomegalovirus (CMV) in patients with Kaposi's Sarcoma
(KS), AIDS Related Complex (ARC), Acquired Immune
Deficiency Syndrome (AIDS), or Lymphoma.

10 Figure 16 demonstrates the detection of
Cytomegalovirus (CMV) in seropositive (Ab+), asymptomatic,
high risk individuals by In Situ Hybridization.

15 Figure 17 demonstrates the detection by In Situ
Hybridization of four different portions of HIV (GAG, ENV,
TAT, LTR) in a person who is at risk for viral infection
but tests sero-negative for HIV.

Figure 18 demonstrates the confirmation of the In
Situ Hybridization results in Figure 17 by a Southern Blot
assay.

20 Figure 19 demonstrates the ability to monitor the
results of alpha-interferon therapy in patients by In Situ
Hybridization.

Figure 20 demonstrates the ability to monitor the
results of gamma-interferon therapy in patients by In Situ
Hybridization.

25

DETAILED DESCRIPTION OF THE INVENTION

Mounting Cells/Tissues

30 The first step in the in situ hybridization
procedure is the deposition of specimens onto a solid
support. Specimens constitute any material which is
composed of or contains cells or portions of cells. The
cells may be living or dead, so long as the target
biopolymer (DNA, RNA or protein) is unaltered and
undamaged and capable of detection. The specimen should
contain cells with substantially intact membranes.

35

1 Although it is not necessary that all membranes of the
cellular structure be intact, the membranes must be
sufficiently preserved to allow: retention of the target
biopolymer, introduction of the detecting probe to the
5 site of the target biopolymer and preservation of
antigenicity of any target membrane components.

10 Techniques for depositing the specimens on the
solid support will depend upon the cell or tissue type and
may include, for example, standard sectioning of tissue or
smearing or cytocentrifugation of single cell
suspensions.

15 Many types of solid supports may be utilized to
practice the invention. Supports which may be utilized
include, but are not limited to, glass, Scotch tape (3M),
nylon, Gene Screen Plus (New England Nuclear) and
nitrocellulose. Most preferably glass microscope slides
are used. The use of these supports and the procedures
for depositing specimens thereon will be obvious to those
of skill in the art. The choice of support material will
depend upon the procedure for visualization of cells and
the quantitation procedure used. Some filter materials
20 are not uniformly thick and, thus, shrinking and swelling
during in situ hybridization procedures is not uniform.
In addition, some supports which autofluoresce will
25 interfere with the determination of low level
fluorescence. Glass microscope slides are most
preferable as a solid support since they have high
signal-to-noise ratios and can be treated to better retain
tissue.

30 Fixation of Cells/Tissues

35 After depositing cells or sections on solid
supports, the samples are fixed. A fixative may be
selected from the group consisting of any precipitating
agent or cross-linking agent used alone or in combination,
and may be aqueous or non-aqueous. The fixative may be

1 selected from the group consisting of formaldehyde
solutions, alcohols, salt solutions, mercuric chloride
sodium chloride, sodium sulfate, potassium dichromate,
potassium phosphate, ammonium bromide, calcium chloride,
5 sodium acetate, lithium chloride, cesium acetate, calcium
or magnesium acetate, potassium nitrate, potassium
dichromate, sodium chromate, potassium iodide, sodium
iodate, sodium thiosulfate, picric acid, acetic acid,
paraformaldehyde, sodium hydroxide, acetones, chloroform,
10 glycerin, thymol, etc. Preferably, the fixative will
comprise an agent which fixes the cellular constituents
through a precipitating action and has the following
characteristics: the effect is reversible, the cellular
morphology is maintained, the antigenicity of desired
15 cellular constituents is maintained, the nucleic acids are
retained in the appropriate location in the cell, the
nucleic acids are not modified in such a way that they
become unable to form double or triple stranded hybrids,
and cellular constituents are not affected in such a way
20 so as to inhibit the process of nucleic acid hybridization
to all resident target sequences. Choice of fixatives and
fixation procedures can affect cellular constituents and
cellular morphology; such effects can be tissue specific.
Preferably, fixatives for use in the invention are
25 selected from the group consisting of ethanol,
ethanol-acetic acid, methanol, and methanol-acetone which
fixatives afford the highest hybridization efficiency with
good preservation of cellular morphology.

Fixatives most preferable for practicing the
30 present invention include 95% ethanol/5% acetic acid for
HL-60 and normal bone marrow cells, 75% ethanol/20% acetic
acid for K562 and normal peripheral blood cells, 50%
methanol/50% acetone for fibroblast cells and normal bone
marrow cells, and 10% formaldehyde/90% methanol for
35 cardiac muscle tissue. These fixatives provide good

1 preservation of cellular morphology and preservation and
accessibility of antigens, and high hybridization
efficiency. According to the present invention, one or
two fixatives for each tissue type are provided which
5 ensure both the best spatial resolution of cells and the
optimal hybridization efficiency.

Simultaneously, the fixative may contain a
compound which fixes the cellular components by
cross-linking these materials together, for example,
10 glutaraldehyde or formaldehyde. While this cross-linking
agent must meet all of the requirements above for the
precipitating agent, it is generally more "sticky" and
causes the cells and membrane components to be secured or
sealed, thus, maintaining the characteristics described
15 above. The cross linking agents when used are preferably
less than 10% (v/v).

Cross-linking agents, while preserving
ultrastructure, often reduce hybridization efficiency;
they form networks trapping nucleic acids and antigens and
rendering them inaccessible to probes and antibodies.
20 Some also covalently modify nucleic acids preventing later
hybrid formation.

Storage of Cells/Tissues

After fixation, microscope slides containing
25 cells may be stored air dried at room temperature for up
to three weeks, in cold (4°C) 70% ethanol in water for
6-12 months, or in paraplast for up to two years. If
specimens are handled under RNase free conditions, they
can be dehydrated in graded alcohols and stored for at
30 least 5 months at room temperature.

Prehybridization Treatments

According to the present invention no formal
35 prehybridization step is necessary. Blocking nonspecific
binding of probe and facilitating probe entry can be
accomplished in the hybridization solution. If short

1 hybridizations are to be done (> 30 min.), slides may be
preheated to hybridization temperature before addition of
the hybridization solution.

Hybridizations

5 Nucleic acid hybridization is a process where two
or more mirror images or opposite strands of DNA, RNA,
oligonucleotides, polynucleotides, or any combination
thereof recognize one another and bind together through
the formation of some form of either spontaneous or
10 induced chemical bond, usually a hydrogen bond. The
degree of binding can be controlled based on the types of
nucleic acids coming together, and the extent of "correct"
binding as defined by normal nucleic acids coming
together, and the extent of "correct" binding as defined
15 by normal chemical rules of bonding and pairing. For
example, if the binding of two strands forms 9 out of 10
correct matches along a chain of length 10, the binding is
said to be 90% homologous.

20 Cellular nucleic acid sequences are detected by
the process of molecular hybridization. The probe must be
"labeled" in some way so to allow "detection" of any
complementary cellular nucleic acid sequences present
within the individual cells.

25 In the present invention, the term
"hybridization" also means the binding of an antibody to a
target antigen.

Types of Probes

30 A probe is defined as genetic material DNA, RNA,
or oligonucleotides or polynucleotides comprised of DNA or
RNA and antibodies. The DNA or RNA may be composed of the
bases adenine, uridine, thymidine, guanine, cytosine, or
any natural or artificial chemical derivatives thereof.
The probe is capable of binding to a complementary or
35 mirror image target cellular genetic sequence through one
or more types of chemical bonds, usually through hydrogen

1 bond formation. The extent of binding is referred to as
the amount of mismatch allowed in the binding or
hybridization process; the extent of binding of the probe
to the target cellular sequences also relates to the
degree of complementarity to the target sequences. The
5 size of the probe is adjusted to be of such size that it
forms stable hybrids at the desired level of mismatch;
typically, to detect a single base mismatch requires a
probe of approximately 12-50 bases. Larger probes (from
10 50 bases up to tens of thousands of bases) are more often
used when the level of mismatch is measured in terms of
overall percentage of similarity of the probe to the
target cellular genetic sequence. The size of the probe
may also be varied to allow or prevent the probe from
15 entering or binding to various regions of the genetic
material or of the cell. Similarly, the type of probe
(for example, using RNA versus DNA) may accomplish these
objectives. The size of the probe also affects the rate
of probe diffusion, probability of finding a cellular
20 target match, etc. Typically, double-stranded DNA
(dsDNA), single-stranded DNA (ssDNA) or RNA probes are
used in a hybridization reaction when nucleotide sequences
are the target.

25 Nucleic acid probes can be prepared by a variety
of methods known to those of skill in the art. Purified
double-stranded sequences of DNA (dsDNA) can be labeled
intact by the process of nick translation or random primer
extension. The ability of double-stranded probes to
hybridize to nucleic acids immobilized within cells is
30 compromised by the ability of the complementary strands to
hybridize to each other in solution prior to hybridization
with the cellular nucleic acids. Single-stranded DNA
(ssDNA) probes do not suffer this limitation and may be
produced by the synthesis of oligonucleotides, by the use
35 of the single-stranded phage M13 or plasmid derivatives of

1 this phage, or by reverse transcription of a purified RNA
template. The use of single-stranded RNA (ssRNA) probes
in hybridization reactions potentially provides greater
signal-to-noise ratios than the use of either double or
5 single-stranded DNA probes. Regardless of whether a
dsDNA, a ssDNA, or a ssRNA probe is used in the
hybridization reaction, there must be some means of
detecting hybrid formation. The means of detecting hybrid
formation utilizes a probe "labeled" with some type of
10 detectable label.

15 Antibody probes are known to those skilled in the
art. The term "antibody probe" means an antibody that is
specific for and binds to any target antigen. Such a
target antigen may be a peptide, protein, carbohydrate or
any other biopolymer to which an antibody will bind with
specifity.

Detection Systems

20 Detectable labels may be any molecule which may
be detected. Commonly used detectable labels are
radioactive labels including, but not limited to, ³²P,
¹⁴C, ¹²⁵I; ³H and ³⁵S. Biotin labeled nucleotides
can be incorporated into DNA or RNA by nick translation,
enzymatic, or chemical means. The biotinylated probes are
detected after hybridization using avidin/streptavidin,
25 fluorescent, enzymatic or colloidal gold conjugates.
Nucleic acids may also be labeled with other fluorescent
compounds, with immunodetectable fluorescent derivatives
or with biotin analogues. Nucleic acids may also be
labeled by means of attaching a protein. Nucleic acids
30 cross-linked to radioactive or fluorescent histone H1,
enzymes (alkaline phosphatase and peroxidases), or
single-stranded binding (ssB) protein may also be used.
To increase the sensitivity of detecting the colloidal
gold or peroxidase products, a number of enhancement or

1 amplification procedures using silver solutions may be
used.

An indirect fluorescent immunocytochemical procedure may also be utilized (Rudkin and Stollar (1977) 5 *Nature* 265: 472; Van Prooijen, et al (1982) Exp.Cell.Res. 141: 397). Polyclonal antibodies are raised against RNA-DNA hybrids by injecting animals with poly(rA)-poly(dT). DNA probes were hybridized to cells in situ and hybrids were detected by incubation with the antibody to RNA-DNA hybrids. 10

According to the present invention RNA probes are preferable to DNA probes (5-8 fold more efficient). Labeling probes with PhotobiotinTM instead of biotin increased the sensitivity of the assay another 2-3 fold. 15

Probe Size and Concentration

The length of a probe affects its diffusion rate, the rate of hybrid formation, and the stability of hybrids. According to the present invention, small probes (50-150 bases) yield the most sensitive, rapid and stable system. A mixture of short probes (50-150 bases) are prepared which span the entire length of the target biopolymer to be detected. For example, if the target biopolymer were 1000 bases long, about 10-20 "different" probes of 50-100 bases would be used in the hybrid solution to completely cover all regions of the target biopolymer. 20 25

The concentration of the probe affects several parameters of the in situ hybridization reaction. High concentrations are used to increase diffusion, to reduce the time of the hybridization reaction, and to saturate the available cellular sequences. According to the 30 present invention, the reaction is complete after 30 minutes (see Figure 2). To achieve rapid reaction rates while maintaining high signal-to-noise ratios, probe concentrations of 2.5-5.0 ug/ml are preferable. Most 35

1 preferable is use of probes at a concentration of 2.5
ug/ml.

Hybridization Solution and Temperature

5 In a preferred embodiment, the hybridization solution of the present invention consists of 50% formamide, 4X SSC (1X SSC = 0.15M sodium chloride and 0.015M sodium citrate), about 0.1M sodium phosphate (pH 7.4), about 100 ug/ml low molecular weight DNA, 0.1% Triton X-100 and about 10-20mM vanadyl ribonucleoside complexes. Single-stranded RNA probe is added to this 10 solution. The probe may be at least 15-20 bases, preferably, 75-150 bases, and labeled with PhotobiotinTM. As shown in Figure 1 the most preferable optimal temperature of hybridization is 50°-55°C. 15 However, temperatures ranging from 15°C. to 80°C. may be used.

Post-Hybridization Treatments and Detections

20 The present invention does not require wash steps prior to hybrid detections. Instead, avidin or streptavidin fluorescent, enzymatic or colloidal gold complexes may be added directly to the slides containing hybridization cocktail and incubated for 20 minutes at room temperature, or 10 minutes at 37°C. This step 25 constitutes a significant advantage over prior hybridization techniques due to the time saved by eliminating several post-hybridization washing steps and the necessary re-blocking of non-specific avidin/streptavidin binding sites; it results in no decrease in signal or increase in noise.

30 The streptavidin/avidin detection step is followed by washes in large volumes of 2x SSC/0.1% Triton X-100. The solution may contain RNase A and T1 at room temperature. This wash can be very short (less than 5 minutes) as long as a continuous gentle circulation or 35 stream of sufficient volume (about 1-200 ml per cm² area

1 of cells) of solution passes over the cells. This may be
followed by washes at higher stringency (lower salt
concentrations such as at least 0.1x SSC and/or higher
temperatures up to 65° C.). Leaving the cell area moist,
5 supports are then dried and coverslipped by any
conventional method.

10 Analysis of the Results of In Situ Hybridizations
Speed, Sensitivity and Quantitation of In Situ
Hybridizations

15 The method of the present invention requires 2-4
hours to complete with a sensitivity of as few as 1-5
molecules of target biopolymers per cell. This results
from the combination of at least three factors: 1)
cellular constituents are not irreversibly precipitated
onto the nucleic acids, 2) the fixation was optimized for
the particular tissue used, and 3) the kinetics of the
reaction proceed more rapidly at high probe concentrations
and at elevated temperatures.

20 The number of copies of mRNA per cell can be
estimated from the number of grains over cells when
radioactive probes are used. With fluorescent or
enzymatic detections a relative estimate of fluorescence
or precipitated colored products allows estimation of mRNA
copy number. Usually, the approximation of copy number is
25 easier after manual photography, film processing and
comparisons of photographic prints.

30 The quantitation of radioactive or fluorescent
signals obtained after in situ hybridizations may be
automated by use of an image analysis system, such as the
Meridian ACAS 470 workstation as is demonstrated in
Example 11.

35 Simultaneous Detection of Three mRNAs

The present invention allows simultaneous
detection of different substances (mRNAs and proteins)

1 within the same cells. This may be accomplished in one of
two ways. First, multiple probes each containing a unique
label (for example, fluorescent tags "A", "B" and "C"
which each emit light at a different detectable wave
length) are all added together in the hybridization
5 solutions. Alternatively, a hybridization and detection
reaction may be carried out with one probe and label,
residual unreacted probe and label washed away under
nuclease-free conditions, and another hybridization
reaction is carried out. This process is repeated as many
10 times as desired. Example 9 demonstrates one embodiment
of the detection of multiple target biopolymers in the
same cell.

15 The following examples are offered by way of
illustration and are not intended to limit the invention
in any manner. In all examples, all percentages are by
weight if for solids and by volume if for liquids, and all
temperatures are in degrees Celcius unless otherwise
noted.

20

EXAMPLE 1

Preparation of Probes.

25 A. General.

RNA or DNA probes useful in the present
invention may be prepared according to methods known to
those of skill in the art or may be obtained from any
commercial source. RNA probes may be prepared by the
methods described by Green et al. (1981) Cell 32:681. DNA
probes may be prepared by methods known to those of skill
30 in the art such as described by Rigby et al. (1977) J.
Mol. Biol. 113:237. Synthetic oligonucleotide probes may
be prepared as described by Wallace, et al (1979) Nucleic
Acids Research 6:3543. The probes useful in the present
35

invention must have the following characteristics:

- 1 1. Specificity for the target
 molecule, and
- 5 2. At least 15 base pairs in length
 and preferably 75-150 base pairs.

5 B. Preparation of RNA probes.

Sub genomic fragments of the c-myc, c-sis,
or c-abl genes were obtained from Amersham Inc. (Catalogue
nos. RPN.1315X, RPN.1324X, and RPN.1325X, respectively).
10 In one embodiment of the present invention, sense strand
probe of the c-myc, c-abl and c-sis genes were utilized.
The c-myc probe used was a 1.3 kb ClaI/EcoRI genomic clone
from the 3' end of the c-myc gene (Dalla-Favera, et al.
(1983) Science 219:963). The c-abl probe was derived from
15 a subclone of the human c-abl gene, an EcoRI/Bam HI
fragment corresponding to the 5' c-abl hybridizing region
(de Klein et al. (1982) Nature 300:765). The c-sis probe
was a Bam HI fragment of clone L33 corresponding to the 3'
end of c-sis (Josephs et al. (1983) Science 219:503). The
HIV and EBV probes were obtained from and prepared as
20 described in Dewhurst, et al. (1987) FEBS Lett. 213:133.
The CMV probe was described in Gronczol, et al. (1984)
Science 224:159. These template plasmid DNAs were
transcribed as described by Green et al. (1981) Cell 32:
25 681. The size and quantity of the RNA was confirmed by
electrophoresis through a denaturing agarose gel as
described by Thomas (1980) Proc. Nat. Acad. Sci. USA 77:
5201 and spectrophotometric measurement performed at A260
and A280. A DNA beta-actin probe prepared as described in
30 Cleveland, et. al. (1980) Cell 20:95 and the RNA probes
were labeled with Photobiotin™ as described by Bresser
and Evinger-Hodges (1987) Gene Anal. Tech. 4: 89,
incorporated herein by reference.

35 Low-molecular weight DNA was added at a
concentration of 100 times that of the probe, and all

1 polynucleotides were precipitated by the addition of 1/3
vol. 10M ammonium acetate and 2-1/2 vol. of 95% ethanol.
The nucleic acids were recovered by centrifugation and
resuspended in water at a concentration of 1 microgram
5 (ug)/microliter (ml) of probe and stored at -70°C until
used.

C. Preparation of Antibody Probes

10 Antibody probes specific for antigens such as viruses or specific determinants thereof, peptides and proteins derived from a variety of sources, carbohydrate moieties and a wide variety of biopolymers are known to those of skill in the art. The methods for preparation of such antibodies are also known to those of skill in the art.

15 Briefly, polyclonal antibodies may be prepared by immunization of an animal host with an antigen. Preferably, the antigen is administered to the host subcutaneously at weekly intervals followed by a booster dose one month after the final weekly dose. Subsequently, 20 the serum is harvested, antibodies precipitated from the serum and detectably labeled by techniques known to those of skill in the art.

25 Monoclonal antibodies may be prepared according to any of the methods known to those in the art. Fusion between myeloma cells and spleen cells from immunized donors has been shown to be a successful method of producing continuous cell lines of genetically stable hybridoma cells capable of producing large amounts of monoclonal antibodies against target antigens such as, for instance, tumors and viruses. Monoclonal antibodies may be prepared, for instance, by the method described in U.S. Patent No. 4,172,124 to Koprowski, et al. or according to 30 U.S. Patent No. 4,196,265 to Koprowski, et al.

35 Procedures for labeling antibodies are known to those of skill in the art.

1

EXAMPLE 2.

Temperature effect on Hybridization.

5 K562 cells (ATCC # CCL 243) were grown in Hank's
Balanced Salt Solution supplemented with 10% fetal calf
serum. Dividing cells were deposited onto glass slides by
cytocentrifugation. Cells were fixed with 75% ethanol,
20% glacial acetic acid, 5% water for 20 minutes at room
temperature. No prehybridization step was performed.
10 Twenty microliters of hybridization solution consisting of
50% formamide, 4x SSC, 0.1M sodium phosphate (pH 7.4),
0.1% Triton X-100, 100 ug/ml low molecular weight DNA
(sheared herring sperm DNA obtained from Sigma Chemical
15 Company) and 2.5 ug/ml of either c-myc, c-abl or c-sis
anti-sense RNA probe labeled with Photobiotin" was added
to each specimen. The anti-sense RNA probes were prepared
as described in Example 1. The hybridization reactions
were carried out at various temperatures ranging from 4°
20 to 80° C. After incubation at the desired temperatures
for two hours, hybrid formation was detected. To detect
hybridization, streptavidin fluorescein or rhodamine
complexes at 2x the manufacturer's recommended concentration
was added to this specimen. After incubation at room
25 temperature for 30 min the specimens were then gently
washed with 1 to 200 ml per centimeter square of cell area
with each of the following solutions containing 0.1%
Triton X-100, in order: 2xSSC, 1xSSC, 0.5xSSC and
0.1xSSC. One drop of a 50/50 (v/v) 100% glycerol/2x PBS
30 solution was added to each specimen. Using a Nikon
fluorescent microscope with photomultipler tube
attachments the fluorescence emitted per cell was recorded
on each slide hybridized at a different temperature.
Approximatley 300 to 800 cells were analyzed per slide.
35 Numerical results obtained indicating the amount of

1 fluorescence from each cell were graphically represented
as relative fluorescence verses the temperature of
hybridization.

5 The results shown of Figure 1 demonstrate that
hybridization temperatures of 50°C to 55°C yields the most
relative fluorescence corresponding to the most hybrid
formation in the present in situ hybridization invention.

10

EXAMPLE 3.

Kinetics of In Situ Hybridization.

15 Figure 2 shows the relationship between the time
of hybridization and the amount of fluorescent signal seen
over cells. K562 cells (ATCC # CCL 243) were grown in
Hank's Balanced Salt Solution supplemented with 10% fetal
calf serum. Dividing cells were deposited onto glass
slides by cytocentrifugation. Cells were fixed with 75%
ethanol, 20% glacial acetic acid, 5% water for 20 minutes
at room temperature. No prehybridization step was
performed. Twenty microliters of hybridization solution
20 consisting of 50% formamide, 4x SSC, 0.1M sodium phosphate
(pH 7.4), 0.1% Triton X-100, 100 ug/ml low molecular
weight DNA (sheared herring sperm DNA obtained from Sigma
Chemical Company) and 2.5 ug/ml of either c-myc, c-abl or
25 c-sis anti-sense RNA probe labeled with PhotobiotinTM
was added to each specimen. The anti-sense RNA probes
were prepared as described in Example 1. The
hybridization reactions were carried out at various times
ranging from 5 minutes to 96 hours. After incubation at
30 55°C. for the desired time, hybrid formation was
detected. To detect hybridization, streptavidin
fluorescein or rhodamine complexes at 2x the manufacturers
concentration were added to the specimen. After
incubation at room temperature for 30 minutes the
specimens were then gently washed with 0.1x SSC/0.1%

35

1 Triton X-100 at 1-200 ml per cm² of cell area. One drop
of a 50/50 (v/v) 100% glycerol/2x PBS solution was added
to each specimen. Using a Nikon fluorescent microscope
5 with photomultiplier tube attachments, the fluorescence
emitted per cell was recorded on each slide hybridized at
each different time point. Approximately 300 to 800 cells
were analyzed per slide. Numerical results obtained
indicating the amount of fluorescence from each cell were
graphically represented as relative fluorescence versus
10 the time of hybridization. Figure 2 demonstrates that the
hybridization reaction is essentially complete after 30
minutes under the conditions of the present invention.

EXAMPLE 4.

15 Changes In Secondary Structure Of Cellular RNA.

HL60 cells (ATCC # CCL 240) were grown in Hank's
Balanced Salt Solution (BSS) supplemented with 10% fetal
calf serum. Cells were harvested and deposited onto glass
microscope slides by cytocentrifugation. Cells were air
dried on glass slides and stored at room temperature until
used. Cells are fixed in one of any number of fixatives
for this type of experiment. Typical fixatives would
include 70% ethanol, 95% ethanol/5% glacial acetic acid,
75% ethanol, 20% glacial acetic acid, 100% methanol, 100%
acetone, 50% acetone, 50% methanol, 4% paraformaldehyde,
2% paraformaldehyde, 10% formaldehyde/90% methanol. After
20 cells were fixed in these fixatives at the appropriate
time and temperature, slides were removed from the
fixative and stained with Wright Giemsa or hematoxylin and
eosin by standard laboratory methods. Cell morphology was
assessed by comparing the degree of preservation of
30 morphology after fixation to the morphology prior to
fixation. Fixatives which did not effectively retain
visual morphology were arbitrarily as rated as +1.
Fixatives which effectively retained cellular morphology

1 were arbitrarily rated as between +1 and +4 with the most
 effective morphologic preservation of cellular morphology
 rated at +4. A second evaluation as to the effective
 preservation of cells by these fixatives was carried out
5 when it was desirable to detect cellular antigens. In
 this case, cells were removed from the fixatives and
 incubated with an antibody specific for a particular
 target cellular antigen. Again fixatives which
 effectively maintain antigenicity of cellular components
10 were arbitrarily rated as +4, while fixatives which did
 not effectively maintain perservation of cellular antigens
 were rated lower, the worst as +1. Fixatives which scored
 as +3 or +4 in terms of preservation of cellular
 morphology and/or preservation of cellular antigenicity
15 were then used in the following steps. Fresh slides
 containing untreated cells were fixed in these fixatives
 and were incubated in hybridization solution containing
 50% formamide, 4x SSC, 0.1 M sodium phosphate, (pH 7.4),
 0.1% Triton X-100, 100 ug/ml low molecular DNA (sheared
20 herring sperm DNA obtained from Sigma Chemicial Company).
 No biopolymer probe was included in this solution. The
 cells were incubated in hybridization solution at 50°-55°C
 for 5, 10, 15, 20, 30, 45, 60, 90, and 120 minutes. After
 the completion of this hybridization step, cell samples
25 were washed gently with 1-200 ml per square centimeter of
 cell area with each of the following solutions containing
 0.1% Triton X-100: 2x SSC, 1x SSC, 0.5x SSC, 0.1x SSC.
 The cellular sample was then evaluated as above for
 preservation of cellular morphology and/or preservation of
 cellular antigenicity. The cell sample was then further
30 evaluated by staining the cells with 50 ug/ml of propidium
 iodide. The propidium iodide will stain double stranded
 and single stranded nucleic acids within the cell. When
 this dye stains double stranded or single stranded nucleic
 acids it has a different characteristic emission spectra
35

1 upon ultraviolet excitation. An untreated cell sample on
a slide is also stained. The total amount of emitted
fluorescence is determined on the untreated cell sample
using a Nikon fluorescence microscope with a
5 photomultiplier tube attachment. 300-1000 cells are
recorded as to the total amount of fluorescence generated
from cytoplasmic double stranded RNA content. This
measurement then represents a base line level for the
total fluorescence in the cytoplasm; that is, the total
10 RNA in the cytoplasm and that RNA being present in a 100%
state of double strandedness. The slides which have been
taken through the various fixation and hybridization
procedures and times are similarly analyzed. In all cases
it is important to chose a fixation and hybridization
15 condition and time which will maintain the same quantity
of fluorescence in the cytoplasm of the cell. During
hybridization, the fluorescence emitted from the RNA of
the cytoplasm of the cell due to the staining of the
propidium iodide will change. The emission pattern
20 decreases relative to the double strandedness of the RNA.
Simultaneously, the wave length emisson which is
reflective of the amount of single stranded RNA in the
cytoplasm will begin to increase. When the total
fluorescence in the cytoplasm due to RNA has remained the
25 same and the amount of fluorescence due to the amount of
double stranded RNA in the cytoplasm has decreased
approximately 70% while the amount of fluorescence
corresponding to the single stranded RNA within the
cytoplasm has increased an equal value, then conditions
have been obtained which will allow the detection of 1-5
30 molecules of RNA within the cytoplasm. The time of the
hybridization reaction which was required to obtained this
shift from double stranded to single strandedness of the
RNA in the cytoplasm is reflective of the time necessary
35 for an actual hybridization reaction to detect 1-5

1 molecules per cell of RNA.

Specifically, in Figure 3 the relative amount of double stranded RNA content is graphically represented on the bottom scale. As the RNA in the cytoplasm becomes more double stranded, the more to the right the curves will fall. The greater the shift in the amount of double strandedness to single strandedness of RNA in the cytoplasm, the greater the shift will be of the curves from the right to the left. The vertical axis represents the cell numbers that were counted. In other words if 300-1000 cells were counted, the vast majority of them fell within a particular area of double strandedness. While some cells had more double strandedness and some had less double strandedness, the analysis can be represented as a bell shape curve. On the right hand side of the figure is shown the various treatments carried out. The result of staining untreated cells with propidium iodide is not shown. However, after treating HL60 cells with various fixatives the amount of double strandedness of cellular RNA remained essentially the same. Even if a prehybridization treatment is carried out which includes a protease treatment there is essentially no change in the amount of RNA double strandedness. The curve in Figure 3 corresponding to the protease treatment is in the same location as the curve for the fixation treatment. It has shifted neither left nor right. However, after fifteen minutes in a hybridization solution, the curve representing the amount of RNA double strandedness has shifted at least 70% to the left. This corresponds to a change in at least 70% of the amount of material in the cytoplasm of the cell becoming single stranded. Comparing this graph to Figure 2 indicates that after 15 min. in the hybridization cocktail, not only is 70% of the RNA in the cytoplasm of the cell single stranded, but as seen in Figure 2, 70% of the hybridization reaction is complete.

EXAMPLE 5.Detection of c-myc oncogene.

Balb/c3T3 cells (ATCC #CCL 163) were grown to density arrest in medium on 8-chamber slides (Tissue-Tek, Miles Laboratories). The medium [Hank's Balanced Salt Solution (BSS) supplemented with 10% fetal calf serum (FCS)] was replaced with serum free medium and the cells serum starved overnight. Multiple specimens were then incubated either in the presence or absence of 15% FCS in Hanks BSS for 45 min at 37°C.

Cells were fixed with 50% acetone and 50% methanol for 20 minutes at room temperature.

No prehybridization step was performed. 20 ul of hybridization solution consisting of 50% formamide, 4x SSC, 0.1M sodium phosphate (pH 7.4), 0.1% Triton X-100, 100 ug/ml low molecular weight DNA (sheared herring sperm DNA obtained from Sigma Chemical Co.) and 2.5 ug/ml c-myc anti-sense RNA probe labelled with Photobiotin TM, was added to each specimen. The c-myc antisense RNA probe was prepared as described in Example 1. After incubation for 2 hrs. at 55°C, hybrid formation was detected.

To detect hybrids, a streptavidin fluorescein complex (Guesdon, J. L., et al (1929) J. Histochem. Cytochem. 27.1131) at 2x the manufacturer's recommended concentration (Bethesda Research Laboratories; Catalog #9538SA; recommended concentration: 7.5 ug/ml) was added to the specimen. After incubation at room temperature for 30 minutes, the specimens were gently washed sequentially (1-200 ml per cm² of cell area) with each of the following solutions containing 0.1% Triton-X 100, in order: 2x SSC, 1x SSC, 0.5x SSC, and 0.1x SSC. One drop of a 50/50 (v/v) 100% glycerol/2x PBS solution was added to each specimen. Specimens were photographed with high speed film (Kodak EES135, PS 800/1600) at 1600 ASA for a 20 sec. exposure on a Nikon Photophot microscope at 400x

1 magnification using a standard filter combination for
transmission of fluorescent light.

5 It has been shown by conventional methods (mRNA
dot blots, Northern blots, and solution hybridizations)
that the c-myc oncogene is not expressed in serum starved
cells but 1-10 copies per cell are induced in serum
stimulated cells. (Armelin et al (1984) Nature 310:656).
Cells probed for expression of c-myc mRNA by the in situ
hybridization procedure of the present invention are shown
10 in Figure 4A and Figure 4B. No c-myc mRNA was detected in
serum starved Balb/c 3T3 cells (Figure 4A) while 1 to 10
copies of c-myc mRNA was detected in serum stimulated
cells by the method of the present invention (Figure 4B).

15

EXAMPLE 6.
Detection of Oncogenes in
Peripheral Blood Cells and Bone Marrow Cells.

20

25

Ten ml. of human peripheral blood or 2 ml. of
human bone marrow cells were incubated at 37° C. in a 1.2%
(215 mOs) ammonium oxalate solution to lyse the red blood
cells. The white blood cells were centrifuged at 3,000
rpm for 10 minutes in a clinical centrifuge. The cell
pellet was subsequently washed with 10 ml. PBS and the
pellet was resuspended in PBS. Cells were deposited by
cytocentrifugation onto precleaned glass slides and air
dried for 5 min. The cells were then fixed in 75%
ethanol/ 20% acetic acid for 20 min. at room temperature.

30

35

No prehybridization step was performed. 20 ul of
hybridization solution consisting of 50% formamide,
4x SSC, 0.1M sodium phosphate (pH 7.4), 0.1% Triton X-100,
100 ug/ml low molecular weight DNA (sheared herring sperm
DNA obtained from Sigma Chemical Co.) and 2.5 ug/ml of
either c-myc, c-sis, or c-abl anti-sense RNA probe
labelled with Photobiotin TM, was added to each
specimen. The anti-sense RNA probes were prepared as

1 described in Example 1. After incubation for 2 hrs. at
55°C, hybrid formation was detected.

5 To detect hybrids, streptavidin fluorescein complex at 2x the manufacturer's recommended concentration was added to the specimen. After incubation at room temperature for 30 minutes, the specimens were then gently washed (1-200 ml per cm² of cell area) with each of the following solutions containing 0.1% Triton-X 100, in order: 2x SSC, 1x SSC, 0.5x SSC, and 0.1x SSC. One drop of a 50/50 (v/v) 100% glycerol/2x PBS solution was added to each specimen. Specimens were photographed with high speed film (Kodak EES135, PS 800/1600) at 1600 ASA for 10 20 sec. exposure on a Nikon Photophot microscope at 400x magnification using a standard filter combination for transmission of fluorescent light.

15 Figure 5 depicts the results from in situ hybridization studies on the expression of three different oncogenes in normal bone marrow (BM), normal peripheral blood (PB), or the peripheral blood from patients with chronic myelogenous leukemia (CML). Blood was obtained from these patients when they were either in the chronic stage of the disease or in the blast stage of the disease. In Figure 5, on the left hand side is shown the three different oncogenes which were analyzed, c-sis, 20 c-myc and c-abl. The numbers below the prints for BM or PB indicate the percent of cells in that sample which are expressing the oncogene indicated on the left. To the 25 bottom of each column of prints (titled BM, PB, chronic or blast) is shown a graphic representation of the relative fluorescent intensity obtained after the hybridizations. The relative fluorescent intensity is indicative of the 30 amount of RNA present within each cell and is scored on a per cell basis. CML in the chronic phase is defined as having less than 5% of the cells in the peripheral blood exist as blasts. In reality, we find that 5-10% of the 35

1 cells in the peripheral blood are over expressing the
three genes which were studied. The expression of these
genes is also considerably elevated as compared to either
normal BM or PB as seen both in the prints and below the
5 prints on the graph. In the blast phase of the disease
which is defined as having greater than 35% of the cells
in the peripheral blood exist as blasts, we find that
greater than 70% of the cells are typically expressing the
10 three oncogenes c-sis, c-myc, and c-abl. The expression of
these genes is elevated when compared to normal bone
marrow or normal peripheral blood but is lower than the
expression of these genes on a per cell basis in the
chronic phase of the disease as seen both in the prints
and in the graphic representation.

15

EXAMPLE 7.

Oncogene detection in solid tissue.

20 Four micron thick frozen sections of human
breast tissue obtained from surgically removed biopsy
samples were mounted on precleaned glass slides and fixed
with 50% methanol/50% acetone for 20 min. at room
temperature.

25 Tissue was hybridized for 4 hours by incubation
at 55°C with a hybridization cocktail containing 50%
formamide, 5x SSC, 0.1 M sodium phosphate (pH 7.4) 20 mM
vanadyl ribonucleoside complexes (New England Biolabs),
100 ug/ml of low molecular weight denatured herring sperm
DNA, and 0.1% Triton X-100. Photobiotinylated RNA probes
(prepared as described in Example 1) were added to the
hybridization cocktail at a concentration of 2.5 ug/ml. No
30 probe was added to the blank panel (Figure 6). Hybrids
were detected by adding fluorescein labelled
avidin/streptavidin (A/SA) solutions directly onto the
slides, and incubated for 30 minutes at room temperature.
Slides were washed, coverslipped and photographed as

35

1 described in Example 6.

Figure 6 demonstrates the results of mRNA *in situ* hybridization and the localization of SIS/PDGF-B expression in the epithelial components of fibrocystic disease (Figure 6, panel "SIS") and lactating adenoma (Figure 6, panel "SIS"). *In situ* hybridization with a Photobiotinylated DNA probe demonstrating expression of the actin gene in the stroma as well as in the epithelial cells of fibrocystic disease (Figure 6, panel "ACTIN"). Lower panels show comparable phase contrast microscopic features of the tissue.

EXAMPLE 8.

Detection of HIV in Human Peripheral Blood.

15 Ten ml. of human peripheral blood or 2 ml. of human bone marrow cells were incubated at 37° C. in a 1.2% ammonium oxalate solution to lyse the red blood cells. The white blood cells were centrifuged at 3,000 rpm for 10 minutes in a clinical centrifuge. The cell pellet was subsequently washed with 10 ml PBS and the pellet was resuspended in PBS. Cells were deposited by cytocentrifugation onto precleaned glass slides and air dried for 5 min. The cells were then fixed in 75% ethanol/ 20% acetic acid for 20 min. at room temperature.

20 25 No prehybridization step was performed. 20 ul of hybridization solution consisting of 50% formamide, 4x SSC, 0.1M sodium phosphate (pH 7.4), 0.1% Triton X-100, 100 ug/ml low molecular weight DNA (sheared herring sperm DNA obtained from Sigma Chemical Co.) and 2.5 ug/ml HIV anti-sense RNA probes labeled with PhotobiotinTM, was added to each specimen. The antisense RNA probes were prepared as described in Example 1. After incubation for 2 hrs. at 55°C, hybrid formation was detected.

30 35 To detect hybrids, streptavidin fluorescein complex at 2x the manufacturer's recommended concentration

1 was added to the specimen. After incubation at room
temperature for 30 minutes, the specimens were then gently
washed (1-200 ml per cm² of cell area) with each of the
following solutions containing 0.1% Triton-X 100, in
5 order: 2x SSC, 1x SSC, 0.5x SSC, and 0.1x SSC. One drop
of a 50/50 (v/v) 100% glycerol/2x PBS solution was added
to each specimen. Specimens were photographed with high
speed film (Kodak EES135, PS 800/1600) at 1600 ASA for
20 sec. exposure on a Nikon Photophot microscope at 400x
10 magnification using a standard filter combination for
transmission of fluorescent light.

Figures 7-10 represent the results seen by
identifying HIV in control cell lines derived from
patients with AIDS or from fresh patient samples. In
15 Figure 7 control cell lines either infected with HIV as
indicated by the plus (+) on the left hand side or a
control cell line infected by HTLV I as indicated by the
minus (-) on the left hand side were hybridized to four
different regions of HIV: ENV, GAG, TAT or LTR genes.
Unless otherwise indicated, anti-sense RNA probes were
20 used in the hybridizations. The top four panels indicate
that these four genes can readily identify HIV in infected
cells. The control probes, the GAG, ENV, TAT, LTR sense
probes, do not detect HIV sequences. They are correctly
negative. The anti-sense RNA probes used in the top four
25 panels to detect HIV are specific. They do not cross react
with other viral sequences such as HTLV I, as indicated in
the bottom two panels. Figure 8 indicates that when the
GAG and ENV probes are used to detect HIV in patients with
Kaposis Sarcoma (KS), the virus is readily identified in
30 fresh peripheral blood. The controls which were performed
on this blood, sense strand controls for these same genes
and a Blank (no probe), were, as expected, negative.
These same probes also identified, as shown in Figure 8, a
virus in a patient with AIDS related complex (ARC). The
35

1 controls, Sense strand RNA and Blank, are negative. In
Figure 9, these same anti-sense RNA probes identified HIV
in a patient with AIDS. The controls, Sense and Blank,
were negative. In Figure 10 these probes identified the
5 presence of HIV in an asymptomatic, seropositive (Ab+)
individual. The controls were negative. These probes did
not cross react with and did not detect HIV in uninfected
normal individuals (Figure 10).

10

EXAMPLE 9.

Simultaneous Detection of three mRNAs
in Human Peripheral Blood.

15

20

25

30

35

Fresh peripheral blood from a patient with
chronic myelogenous leukemia in early accelerated phase
was obtained by venipuncture. Red blood cells were lysed
with ammonium oxalate. White blood cells were prepared
and deposited onto slides as described in Example 6.
After fixation, the specimens were taken through the
several hybridization steps as described above in
Example 6 with the following modifications: a probe for
the c-sis gene was incubated with the slides for 1 hour
using the in situ hybridization solution described in
Example 5. Streptavidin-rhodamine was used to detect
hybrid formation. The wash steps followed this detection,
only all solutions were RNase free and contained 0.01M
D-Biotin. A hybridization was repeated with a second
probe for the c-myc gene; after 1 hour, the hybrids formed
were detected using streptavidin-FITC. Washes were
repeated as above, and a final hybridization was carried
out with a probe for the c-abl gene. Hybrids formed with
this probe were detected using streptavidin conjugated to
alkaline phosphatase. The specimens were washed as
described in Example 5 with the inclusion of 1-10 ug/ml of
RNase A to each of the wash solutions. The substrates for
the alkaline phosphatase (nitroblue tetrazolium and

1 5-bromo, 4-chloro, 3-indol phosphate) were added, and the
reduction of nitroblue tetrazolium was carried out for 5
minutes at room temperature.

5 Using this in situ hybridization technique which
permitted the simultaneous detection of multiple different
mRNA species, we have demonstrated that the
over-expression of c-sis, c-myc and c-abl all occur within
the same cells in patients' peripheral blood with chronic
myelogenous leukemia. In Figure 11, the cells containing
10 the c-myc oncogene mRNA (MYC, the left panel) was detected
by the presence of a green color emission due to the
reaction of a streptavidin-fluorescein complex with the
hybrids formed between the probe and target biopolymer
sequences. The same cells were also shown to contain the
15 c-sis oncogene mRNA (SIS, middle panel) by the detection
of red fluorescence resulting from the presence of reacted
avidin/streptavidin-rhodamine with the reacted probe. The
presence of the c-abl oncogene (ABL) within the same cells
is shown in the right panel by the presence of the dark
20 blue, reacted and precipitated nitroblue tetrazolium
product.

25 In Figure 12, the same result is shown as in
Figure 11, only a different patient sample was used and a
different detection method was employed to identify the
presence of the c-abl oncogene mRNA. In this case,
streptavidin labeled with colloidal gold (Bethesda
Research Laboratories, catalog # 9532SA; Horisberger, M.
30 (1981) Scanning Electron Microscopy 11:9) was used in the
detection step described above instead of streptavidin
tagged with the enzyme alkaline phosphatase. No further
treatments were necessary and the cells were washed as
described above. The presence of black precipitate or
grains seen within cells when using either bright field or
phase contrast microscopy techniques, or the visualization
35 of bright white areas of light when using either

1 epi-polarization or dark field microscopy indicated that
the cells contained target biopolymer mRNA sequences
substantially complementary to the probe, in this case to
the c-abl gene.

5

EXAMPLE 10.

Detection of Nucleic Acids and Proteins in
Peripheral Blood Cells.

10 Ten ml. of human peripheral blood were incubated at 37° C. in a 1.2% ammonium oxalate solution to lyse the red blood cells. The white blood cells were centrifuged at 3,000 rpm for 10 minutes in a clinical centrifuge. The cell pellet was subsequently washed with 10 ml. PBS and the pellet was resuspended in PBS. Cells were deposited by cytocentrifugation onto precleaned glass slides and air dried for 5 min. The cells were then fixed in 75% 15 ethanol/20% acetic acid for 20 min. at room temperature.

No prehybridization step was performed. 20 ul of hybridization solution consisting of 50% formamide, 4x 20 SSC, 0.1M sodium phosphate (pH 7.4), 0.1% Triton X-100, 100 ug/ml low molecular weight DNA (sheared herring sperm DNA obtained from Sigma Chemical Co.) and 2.5 ug/ml of c-abl anti-sense RNA probe labelled with Photobiotin TM, was added to each specimen. The anti-sense RNA probe was prepared as described in Example 1. After incubation for 25 2 hrs. at 55°C, hybrid formation was detected.

To detect hybrids, streptavidin rhodamine complex at 2x the manufacturer's recommended concentration was added to the specimen. During this incubation, rabbit 30 polyclonal antibody raised against the c-abl gene product (supplied by Dr. Russel Grieg of Smith Kline & French, Swedeland, Pennsylvania) was added to the specimen at a concentration which positively labeled K562 cells and did not show any detectable signal in HL60 cells when a 35 fluorescein labeled anti-rabbit IgG was added.

After incubation at room temperature for 30 minutes, the specimens were then gently washed (1-200 ml per cm² of cell area) with each of the following solutions containing 0.1% Triton-X 100, in order: 2x SSC, 1x SSC, 0.5x SSC, and 0.1x SSC. One drop of a 50/50 (v/v) 100% glycerol/2x PBS solution was added to each specimen. Specimens were photographed with high speed film (Kodak EES135, PS 800/1600) at 1600 ASA for 20 sec. exposure on a Nikon Photophot microscope at 400x magnification using a standard filter combination for transmission of fluorescent light.

It is known that c-abl mRNA and protein are over-produced within the same cell in patients with chronic myelogenous leukemia (CML) (Stam, K. et al. N. Engl. J. Med. 313: 1429; Konopka, J.B. and Witte, O.N. (1984) Cell 37: 3116). Using the present invention, peripheral blood cells from a patient with CML were probed for the presence of mRNA corresponding to the c-abl gene and simultaneously, as described above, for the presence of the c-abl protein product. Figure 13 A demonstrates that the protein was readily detectable due to the fluorescein fluorescence emission of the reacted antibodies. In the same cells, Figure 13 B shows the presence of the c-abl mRNA, detectable due to the rhodamine fluorescence emission.

EXAMPLE 11.

Quantitation of the Number of Target Biopolymer Molecules.

K562 Cells (ATCC #CCL 243) were grown in Hank's Balanced Salts Solution supplemented with 10% Fetal Calf Serum. Three days after the last change in media, the cells were split to a density of about 10⁵ cells per 0.3 ml. of fresh media. One hour later, 60 replica slides were made by depositing 50,000-100,000 cells onto a slide by cytocentrifugation. The remainder of the cells were

1 harvested and RNA and DNA was extracted from the cells by
the guanidium cesium chloride method (Chirgwin, et al.
(1979) Biochemistry 18: 5294).

5 Since the cell line was a relatively homogeneous population, the extracted RNA was purified and used to determine copy number estimates for each RNA species analyzed; i.e., an estimate could be made of the number of molecules of each gene present within each cell from a series of control experiments well known to those with knowledge and skill in the art. These control experiments 10 to determine the number of molecules per cell included the following: Northern blots, RNA dot blots, Quick-blots™, Cytodots™, single copy saturation experiments, and solution concentration versus time hybridization 15 experiments ($\text{Rot}_{1/2}$ analysis) (Hames, B.D. and Higgins, S.J. (1986) in Nucleic Acid Hybridization: a practical approach, IRL Press, Oxford-Washington, D.C.).

20 Cells on slides were fixed with 75% ethanol/20% glacial acetic acid/5% water for 20 minutes at room temperature.

No prehybridization step was performed. 20 ul of hybridization solution consisting of 50% formamide, 4x SSC, 0.1 M sodium phosphate (pH 7.4), 0.1% Triton X-100, 100 ug/ml low molecular weight DNA (sheared herring sperm 25 DNA obtained from Sigma Chemical Co.) and 2.5 ug/ml of an anti-sense RNA probe labelled with Photobiotin™, was added to each specimen. Probes used were either the sense or anti-sense RNA strands the following genes: c-abl, c-sis, c-myc, or Epstein Barr Virus (EBV). The probes 30 were prepared as described in Example 1. After incubation for 2 hours at 55° C, hybrid formation was detected.

To detect hybrids, streptavidin fluorescein (SA-FlTC), phycoerytherin (SA-PE), rhodamine B(SA-R), Texas Red™ (SA-TR), phycocyanin (SA-PC), or allophycocyanin (SA-APC) complexes were added at 2x the 35

1 manufacturer's recommended concentration. (SA-FITC,
SA-TR: Bethesda Research Laboratories; SA-R: Southern
Biologicals; SA-PE, SA-PC, SA-APC: BioMeada). After
incubation at 37° C for 10 minutes, the specimens were
5 then gently washed with (1-200 ml. per cm² of cell area)
with 0.1x SSC containing 0.1% Triton X-100. One drop of a
50/50 (v/v) 100% glycerol/2x PBS solution was added to
each specimen and a #1 coverslip was placed over the cells
before microscopic examination.

10 Fluorescence emitted from each cell is a
reflection of the number of streptavidin molecules which
reacted with probe; the amount of reacted probe within a
cell is indicative of the number of target biopolymers
present within the cell. To measure the fluorescence
within each cell, slides were analyzed using the ACAS 470
15 Workstation from Meridian Instruments (Okemos, MI). The
Meridian instrument, like most image processing systems,
excites the fluors present within a sample and then
captures the emitted light as either a digital or analog
20 signal. This signal is digital on the Meridian
instrument. The quantity of the signal can be represented
by different colors. In Figure 14, this is illustrated in
the top right hand panel which shows the colors the
instrument assigns to emitted signals of different
25 intensities. When these colors are represented over a cell
(Figs. 14 A-C), the relative amount of emitted
fluorescence per cell can be seen. In Figure 14A shows
the detection of the c-sis gene; the intensity of emission
of reacted fluorescer is seen; in Figure 14B, the
30 detection of c-myc is shown. Figure 14C shows the
background signal emitted when no probe is included in the
hybridization solution. This panel is a negative control
and is blank. The Meridian instrument can determine the
total fluorescence over the entire cell (i.e., quantity of
35 fluorescence per cell) and represent this information

1 graphically. The control experiments described above
which were carried out with purified RNA from other cells
showed that both the c-sis and c-myc cellular target genes
were present in these cells at between 1 and 10 molecules
5 per cell. Therefore, this value represents the scale on
the horizontal axis in Figures 14D and 14E. The present
invention together with the appropriate instrumentation,
was capable of identifying the number of cells which
contained even a single molecule of either the c-sis or
10 c-myc gene.

EXAMPLE 12.

Error Rates of the In Situ Hybridization System.

15 K562 Cells (ATCC #CCL 243) were grown in Hank's
Balanced Salts Solution supplemented with 10% fetal calf
serum. Three days after the last change in media, the
cells were split to a density of about 10^5 cells per 0.3
ml of fresh media. One hour later, 60 replica slides were
made by depositing 50,000-100,000 cells onto a slide by
20 cytocentrifugation. The remainder of the cells were
harvested and RNA and DNA was extracted from the cells by
the guanidium cesium chloride method as in the previous
Example 11.

25 Since the cell line was a relatively homogeneous
population, the extracted RNA was purified and used to
determine copy number estimates for each RNA species
analyzed; i.e., an estimate could be made of the number of
molecules of each gene present within each cell from a
series of control experiments well known to those with
30 knowledge and skill in the art. These control experiments
to determine the number of molecules per cell included the
following: Northern blots, RNA dot blots, Quick-blots™,
Cytodots™, single copy saturation experiments, and
solution concentration versus time hybridization
35 experiments (Rot_{1/2} analysis) (Hames, B.D. and Higgins,

1 S.J. (1986) in Nucleic Acid Hybridization: a practical
approach. IRL Press, Oxford-Washington, D.C.).

5 Cells on slides were fixed with 75% ethanol/20%
glacial acetic acid/5% water for 20 minutes at room
temperature.

10 No prehybridization step was performed. 20 ul of
hybridization solution consisting of 50% formamide, 4x
SSC, 0.1 M sodium phosphate (pH 7.4), 0.1% Triton X-100,
100 ug/ml low molecular weight DNA (sheared herring sperm
DNA obtained from Sigma Chemical Co.) and 2.5 ug/ml of an
15 anti-sense RNA probe labeled with Photobiotin™, was
added to each specimen. Probes used were either the sense
or anti-sense RNA strands of the following genes: c-abl,
c-sis, c-myc, or Epstein Barr Virus (EBV). The probes
were prepared as described in Example 1. After incubation
for 2 hours at 55° C, hybrid formation was detected.

20 To detect hybrids, streptavidin fluorescein
(SA-FITC), phycoerytherin (SA-PE), rhodamine B(SA-R),
Texas Red™ (SA-TR), phycocyanin (SA-PC),
allophycocyanin (SA-APC) complexes were added at 2x the
25 manufacturer's recommended concentration. (SA-FITC,
SA-TR: Bethesda Research Laboratories; SA-R: Southern
Biologicals; SA-PE, SA-AP: BioMeada). After incubation at
37° C for 10 minutes, the specimens were then gently
washed (1-200 ml per cm² of cell area) with 0.1x SSC
30 containing 0. 1% Triton X-100™. One drop of a 50/50
(v/v) 100% glycerol/2x PBS solution was added to each
specimen and a #1 coverslip was placed over the cells
before microscopic examination.

35 Fluorescence emitted from each cell is a
reflection of the number of streptavidin molecules which
reacted with probe; the amount of reacted probe within a
cell is indicative of the number of target biopolymers
present within the cell. To measure the fluorescence
within each cell, slides were analyzed using the ACAS 470

1 Workstation from Meridian Instruments (Okemos, MI). The
Meridian instrument, like most image processing systems,
excites the fluors present within a sample and then
captures the emitted light as either a digital or analog
5 signal. This signal is digital on the Meridian
instrument. In a manner similar to the method described
in Example 11, the total fluorescence per cell was
determined using the ACAS 470 workstation. The data
obtained was then analyzed by the Mann-Whitney test to
determine if there were statistically significant
10 differences between the amounts of fluorescence seen when
different probes were used in the in situ hybridization
system. In a cell line which has a known target
biopolymer RNA present, a probe should react with the
target; this would lead to the generation of a fluorescent
15 signal within the positive cells. In cases in which the
"target" biopolymer RNA is known to be absent from the
cells, a probe reactive to the target should not bind in
any non-specific manner to the cells and thus should not
generate any fluorescent signal within the cells. A
20 statistical test can determine whether this is true and
whether the difference between the "positive" and the
"negative" is sufficiently different to be correct and not
random. Furthermore, the statistical test can determine
the probability of the test incorrectly identifying a
25 negative sample as positive or a positive sample as
negative. Table 1 shows the results of this statistical
analysis. The positive samples were correctly
identified. The error rates represent the chance of
30 obtaining false results when different thresholds of
sensitivity for the present invention are employed.

1

TABLE 1

In Situ Hybridization
False Positive, False Negative Rates

5

	<u>Detection Threshold</u>	<u>Error Rate</u>
	1-2 genes/cell	1.71%
10	1-5 genes/cell	0.65%
	>10 genes/cell	>0.005%

15

EXAMPLE 13.

Detection of Cytomegalovirus in Peripheral Blood.

One ml. of human peripheral blood was obtained from the patients described in Example 8 and processed as described in that Example. The hybridization reaction was carried out with the specimens using the same hybridization cocktail described in Example 4, except the probe was an anti-sense RNA probe complementary to cytomegalovirus (CMV) RNA and labeled with Photobiotin™. Hybrid detection was carried out as described in Example 8.

The column of photomicrographs in Figures 15 and 16 on the left (CMV) illustrates that the present invention is capable of detecting target viral biopolymers --- here detecting CMV --- within a specimen. The presence of CMV in the specimen is indicated by the emitted light within the cells. The column of photomicrographs in Figures 15 and 16 on the right (BLANK) shows no emitted light; these pictures show that in the controls no extraneous signals were produced.

35

EXAMPLE 14.

Detection of HIV in Peripheral Blood Cells
of an Individual Seronegative for HIV
But at High Risk for HIV Infection.

Ten ml. of human peripheral blood from an individual at high risk for HIV infection was obtained, processed, hybridized and hybrids detected and photographed as described in Example 8. In Figure 17, the panels marked GAG, ENV, TAT, LTR, EBV represent results obtained when the corresponding anti-sense RNA probes were added to the hybrid solution. The panels with HIV anti-sense probes added are positive while EBV is negative. The panel marked "Blank" represents results obtained when no probe was added to the hybrid solution and is negative. The bottom right panel is a phase contrast photomicrograph of the cells in the panel marked "Blank".

To confirm that HIV was present in the blood cells of this individual, a Southern blot analysis (Southern, (1975) J. Mol. Biol. 98:503) of DNA from the HIV infected cell line H-9 (ATCC #CRL 8543) (lanes A and C) and from the peripheral blood cells from this same seronegative but high risk individual (lanes B and D) is presented in Figure 19. DNA in lanes A and B was digested with Sst I and in lanes C and D with Hind III. The blot was hybridized with a full length HIV probe, radiolabelled with ³²P, and demonstrates that HIV hybridizing sequences are present in the peripheral blood cells of this individual.

EXAMPLE 15.

Usefulness of In Situ Hybridization
to Monitor Effectiveness of Patient Therapy.

Peripheral blood was obtained from patients with chronic myelogenous leukemia (CML) both before and after

1 treatment with either alpha or gamma interferon. The
blood was processed, hybridization was accomplished, and
hybrids were detected as described in Example 6. Figure
19 demonstrates that in a CML patient before
5 alpha-interferon treatment (Day 0) the c-myc, c-sis, and
c-abl oncogene target biopolymers were all present, as
demonstrated by the light emitted from the cells and seen
on the photomicrographs at day 0. In the same patient,
the same target cellular genes were not produced after
10 four days of alpha-interferon therapy (little or no signal
is seen in the cells at day 4). In contrast, in a patient
who underwent treatment with gamma-interferon, cells were
still present which over-produced the c-sis and c-abl
15 oncogene (Figure 20, Panels E and F). Clinically, the
patient who was treated with alpha-interferon responded
well to the therapy and went into remission. The patient
who received gamma-interferon failed to respond to this
therapy. The monitoring of changes in the type or amount
20 of a cellular target biopolymer sequence may be an
important means of evaluating or predicting the
effectiveness of therapeutics.

One skilled in the art will readily appreciate
that the present invention is well adapted to carry out
the objects and obtain the ends and advantages mentioned,
25 as well as those inherent therein. The components,
methods, procedures and techniques described herein are
presently representative of the preferred embodiments, are
intended to be exemplary, and are not intended as
limitations on the scope of the present invention.
Changes therein and other uses will occur to those skilled
30 in the art which are encompassed within the spirit of the
invention and are defined by the scope of the appended
claims.

35 What is claimed is:

1 1. A method for assaying biopolymers in a
specimen having substantially intact membranes comprising
the steps of:

5 contacting said specimen with a fixation
medium comprising at least one agent selected
from the group consisting of a precipitating
agent and a cross linking agent,

10 contacting said fixed specimen with a
hybridization solution consisting of a denaturing
agent, a hybrid stabilizing agent, a buffering
agent, a selective membrane pore-forming agent
and at least one probe having a nucleotide
sequence at least substantially complementary to
a specific target nucleotide sequence to be
detected, said contacting being under hybridizing
15 conditions at a temperature of 15-80°C for about
20-120 min.,

20 incubating said specimen with said medium in
the presence of at least one detectable label,

25 detecting duplex formation by means of said
label, wherein said method is capable of
detecting as few as 1-5 biopolymers per cell.

25 2. The method of Claim 1 wherein said label is
attached to said probe.

30 3. The method of Claim 1 wherein said label is
added after the duplex formation is complete.

35 4. The method of Claim 1 wherein said label is
selected from the group consisting of radioactive labels,
fluorescers, chemiluminescers, enzyme labels, and
radiolabels.

1 5. The method of Claim 3 wherein said label is
selected from the group consisting of aviden and
streptaviden.

5 6. The method of Claim 1 wherein said
precipitating agent is selected from the group consisting
of ehtanol, methanol, acetone, formaldehyde and
combinations thereof.

10 7. The method of Claim 1 wherein said
cross-linking agent is selected from the group consisting
of paraformaldehyde, formaldehyde, dimethylsilserimidate,
and ethyldimethylamino-propylcarbodiimide.

15 8. The method of Claim 1 wherein said
denaturing agent is selected from the group consisting of
formamide, urea, sodium iodide, thiocyanate, guanidine,
perchlorate, trichloroacetate, and tetramethylamine.

20 9. The method of Claim 1 wherein said hybrid
stabilizing agent is selected from the group consisting of
sodium chloride, lithium chloride, magnesium chloride, and
ferric sulfate.

25 10. The method of Claim 1 wherein said pore
forming agent is selected from the group consisting of
Brij 35, Brij 58, Triton X-100, CHAPSTM, desoxycholate
and dodecyl sulfate.

30 11. The method of Claim 1 wherein said
biopolymer is RNA.

35 12. The method of Claim 1 wherein said
biopolymer is DNA.

1 13. The method of Claim 1 wherein said
biopolymer is an antigen.

5 14. The method of Claim 1 wherein at least two
biopolymers are assayed simultaneously in the same
sample.

10 15. The method of Claim 14 wherein at least one
biopolymer is a polynucleotide and a second biopolymer is
an antigen.

15 16. The method of Claim 1 wherein said
temperature is 15°C- 80°C.

15 17. The method of Claim 16 wherein said
temperature is 50°C to 55°C.

20 18. The method of Claim 1 wherein said method is
accomplished within about 4 hours.

25 19. The method of Claim 1 wherein said
biopolymer is selected from the group consisting of a RNA,
a DNA, a viral gene, an oncogene, and an antigen.

25 20. A method of assaying biopolymers in
peripheral blood and bone marrow cells having
substantially intact membranes comprising the steps of:
 depositing said specimen on a solid support,
 contacting said specimen with a fixation
30 medium comprising 75% ethanol/20% glacial acetic
acid/5% water for at least 10 minutes at a
temperature ranging from -20°C to 50°C,
 contacting said fixed specimen with a
hybridization solution at about 50°-55°C for at
35 least 5 min said hybridization solution

1 comprising about 20-80% formamide, about 5 times
concentrated SSC, about 0.1M TRIS-HCl, pH 7.4,
about 0.1% Triton X-100, and a photobiotinylated
single stranded anti-sense RNA probe having
5 75-150 bases at least substantially complementary
to a specific target nucleotide sequence to be
detected,

10 adding a detectably labeled agent selected
from the group consisting of aviden and
streptavidin at a concentration sufficient to
bind said hybridized probe within at least 5 min,
washing said labeled specimen with 0.1x SSC
containing 0.1% Triton X-100 to remove unbound
labeled agent, and
15 detecting said bound label.

21. The method of claim 20, wherein said
biopolymer is an oncogene.

22. The method of claim 20, wherein said
biopolymer is a virus.

25 23. A method of assaying biopolymers in tissue
samples having substantially intact membranes comprising
the steps of:

30 depositing said tissue specimen on a solid
support,
contacting said specimen with a fixation
medium comprising 50% methanol/50% acetone for at
least 20 minutes at a temperature ranging from
-20°C to 50°C,
35 contacting said fixed specimen with a
hybridization solution at about 50°-55°C for at
least 5 min said hybridization solution

1 comprising about 20-80% formamide, about 5 times
concentrated SSC, about 0.1M sodium phosphate,
pH 7.4, about 0.1% Triton X-100, 20 mM vanadyl
5 ribonucleoside complexes, low molecular weight
denatured DNA at a concentration 100 times
greater than the probe concentration and a
photobiotinylated single stranded anti-sense RNA
probe having 75-150 bases at least substantially
complementary to a specific target nucleotide
sequence to be detected,

10 adding a detectably labeled agent selected
from the group consisting of aviden and
streptavidin at a concentration sufficient to
bind said hybridized probe within at least 5 min,
15 washing said labeled specimen with 0.1 X SSC
containing 0.1% Triton X-100 to remove unbound
labeled agent, and
detecting said bound label.

20

25

30

35

1/20

Temperature Effect on In Situ
Hybridizations

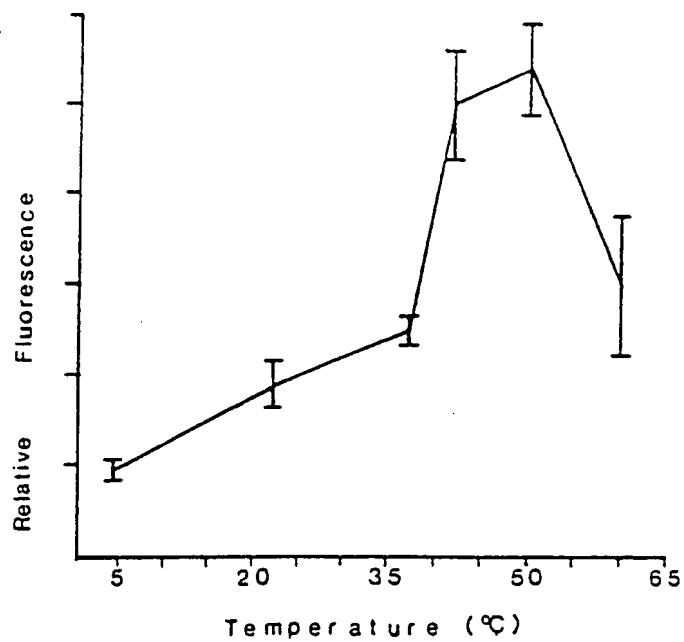


Figure 1

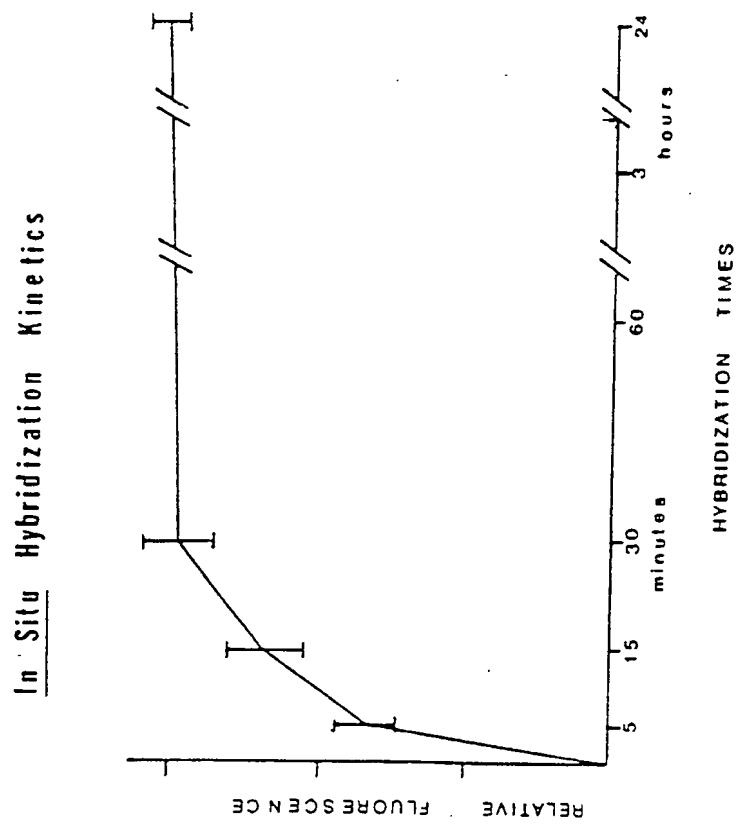


Figure 2

3/20

SECONDARY STRUCTURE OF CELLULAR RNA

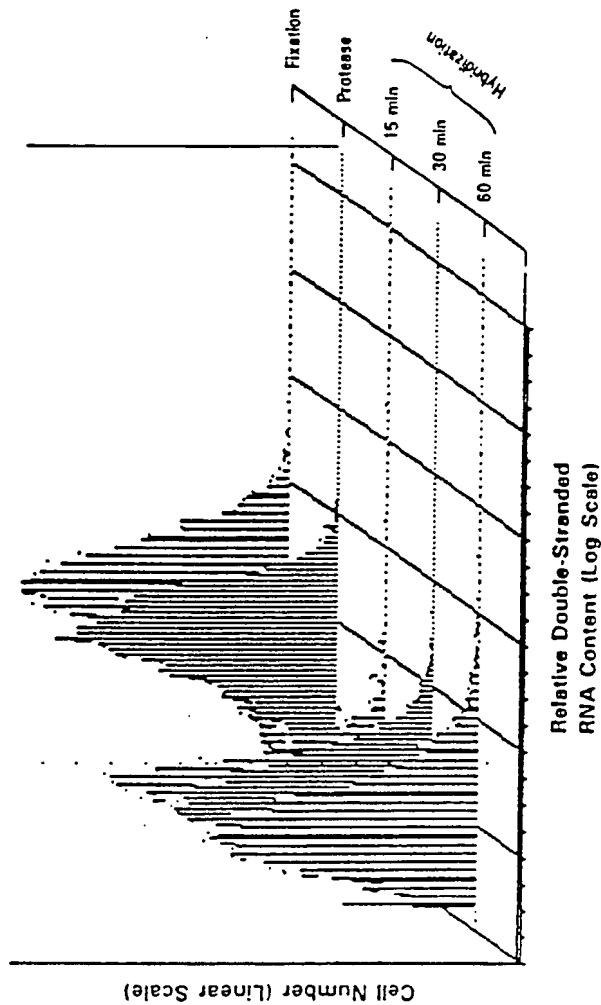


Figure 3

4/20

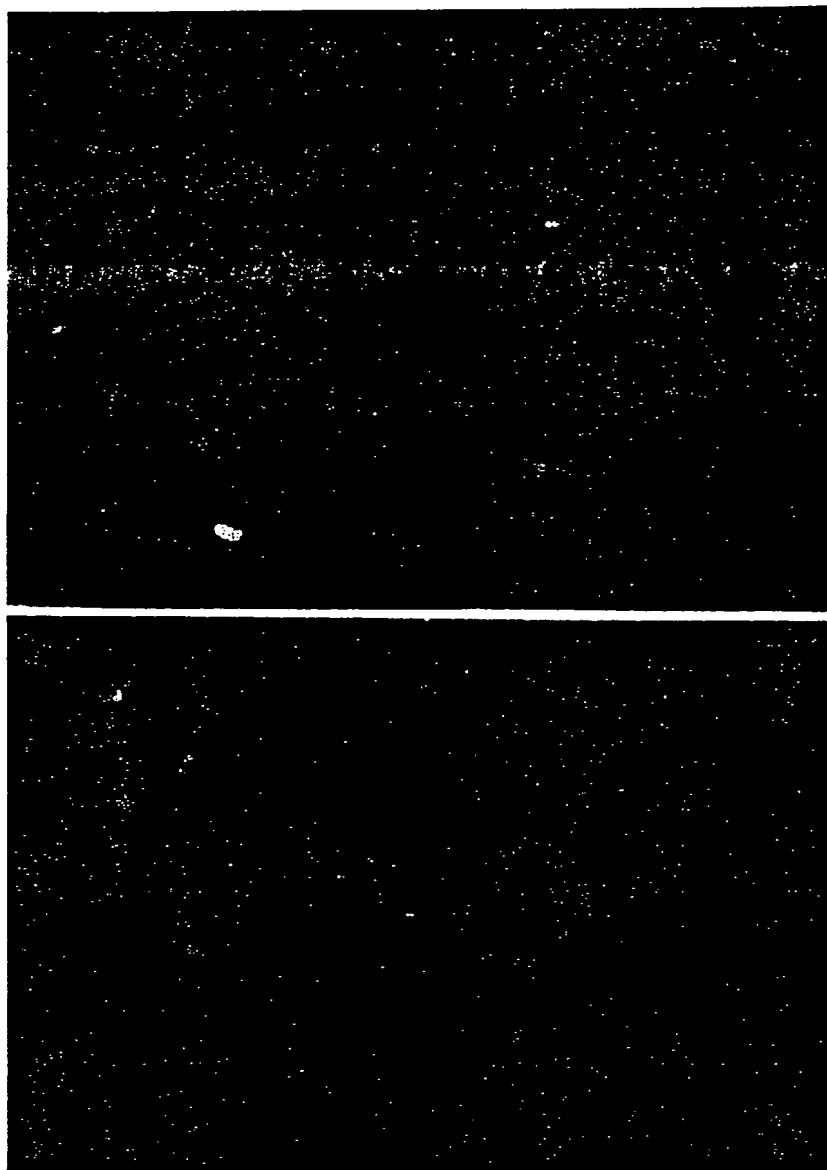


FIG. 4

SUBSTITUTE SHEET

5/20

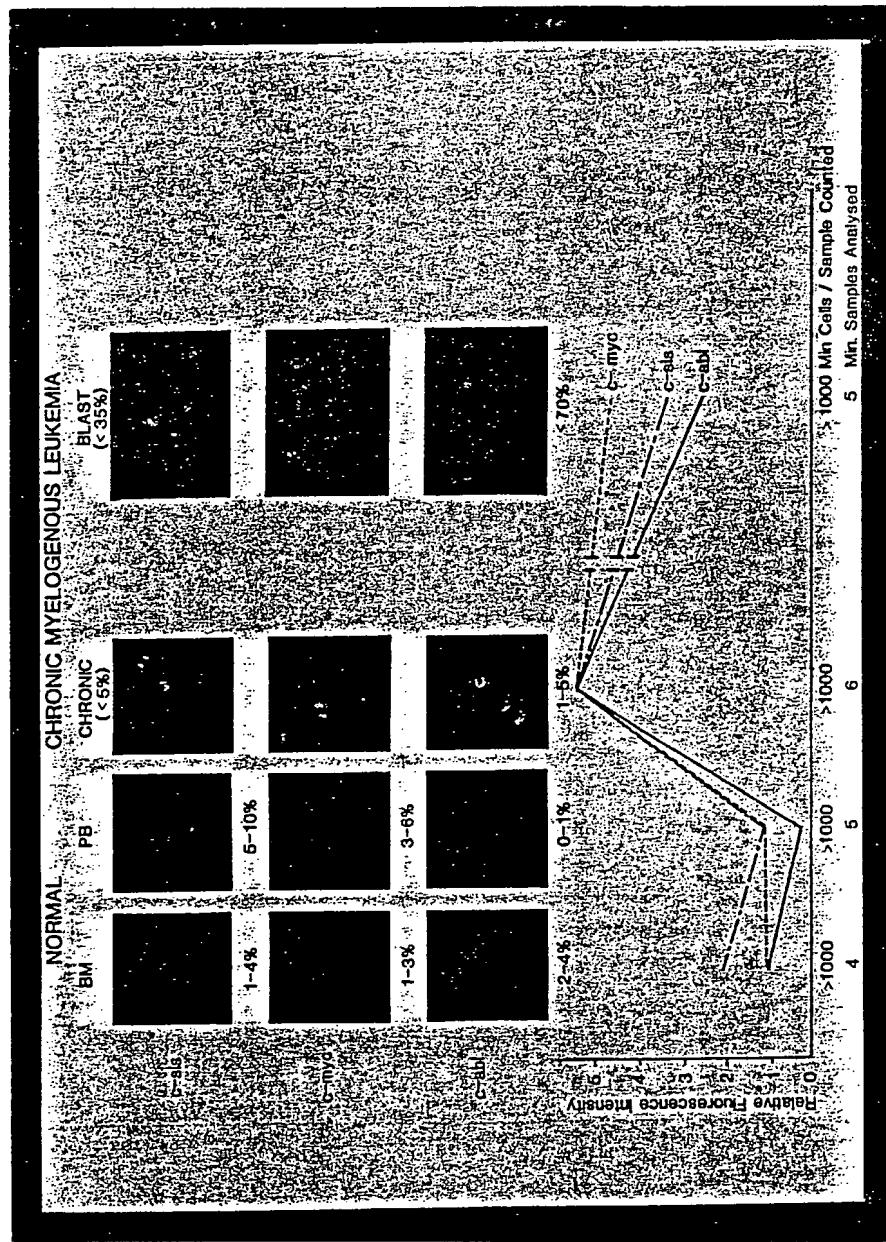


FIG. 5

SUBSTITUTE SHEET

6/20

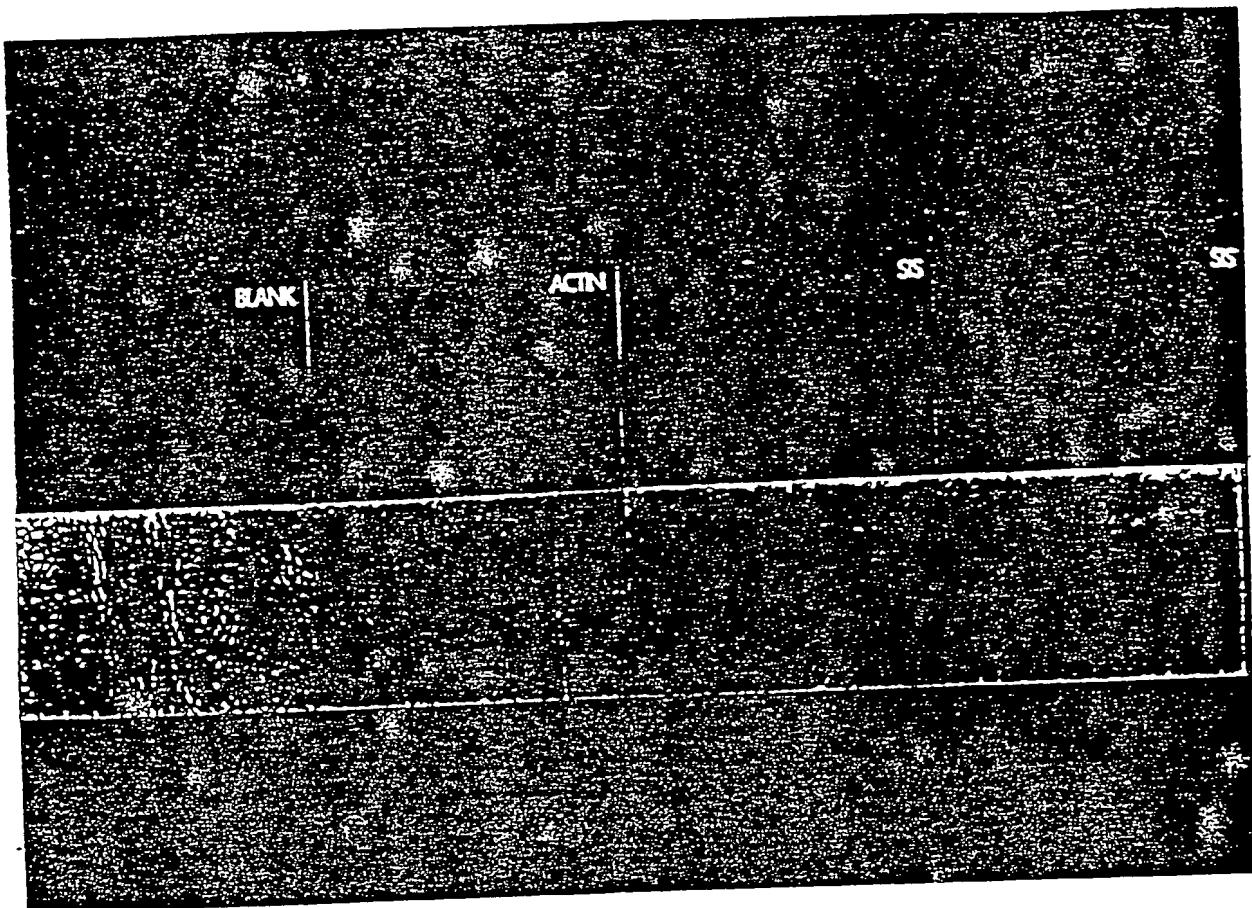


FIGURE 6

7/20

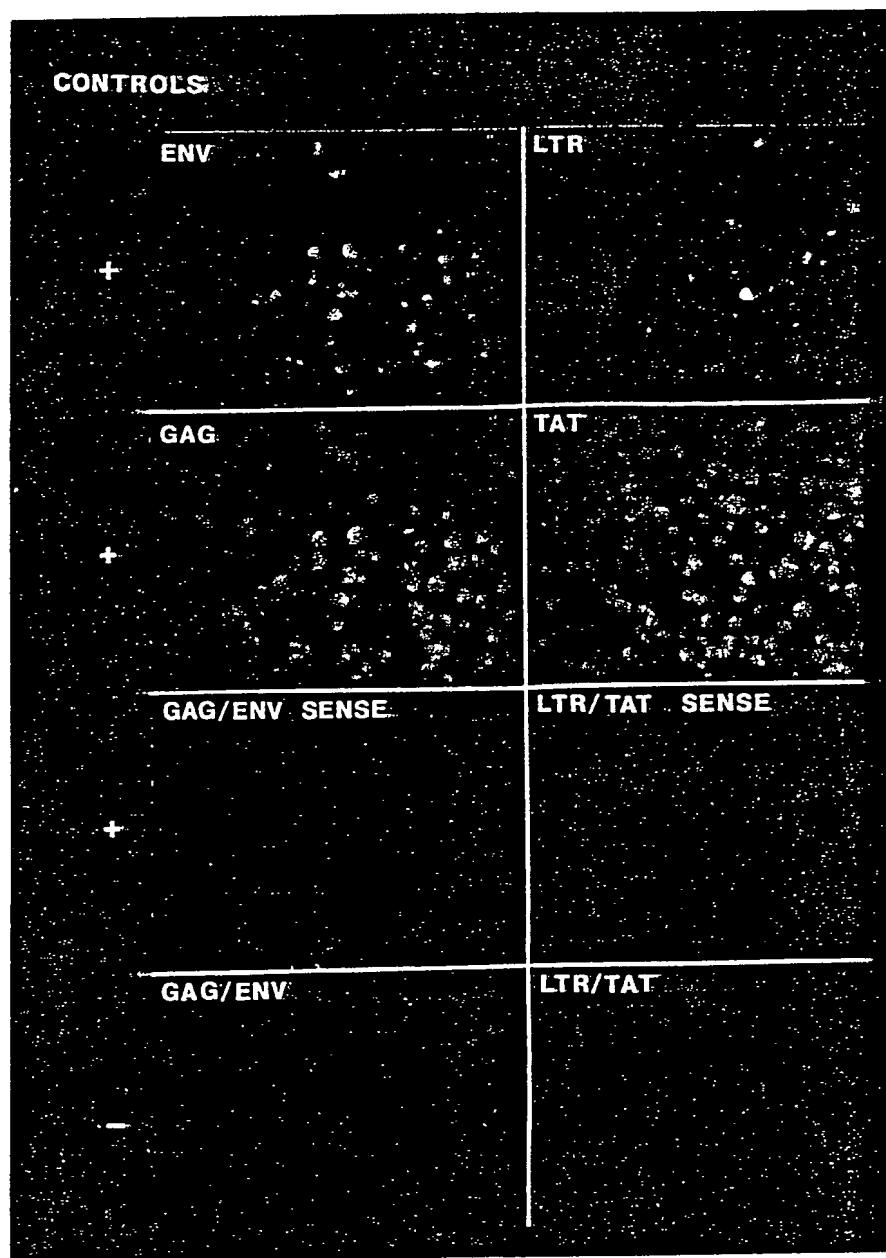


FIG. 7

SUBSTITUTE SHEET

8 | 20

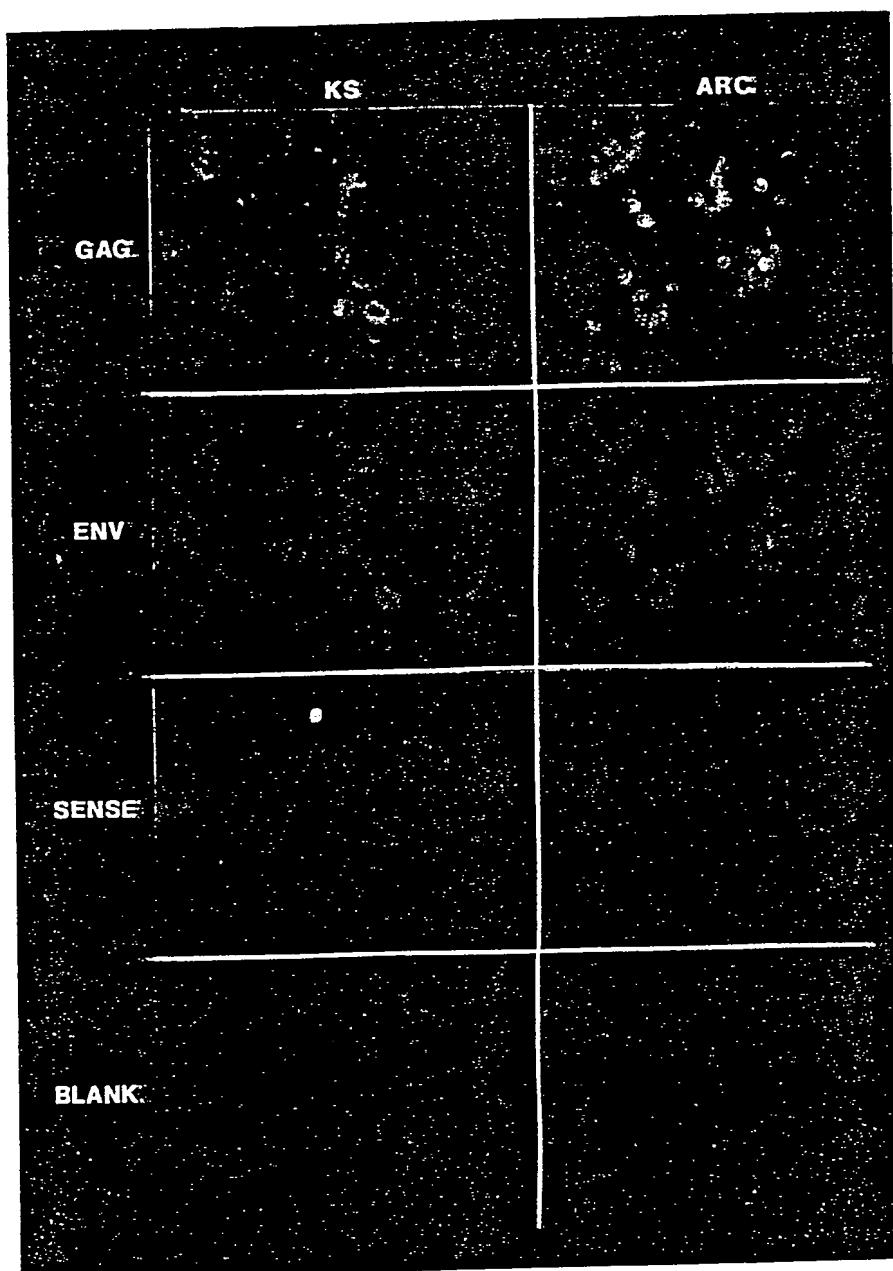


FIG. 8

SUBSTITUTE SHEET

9/20

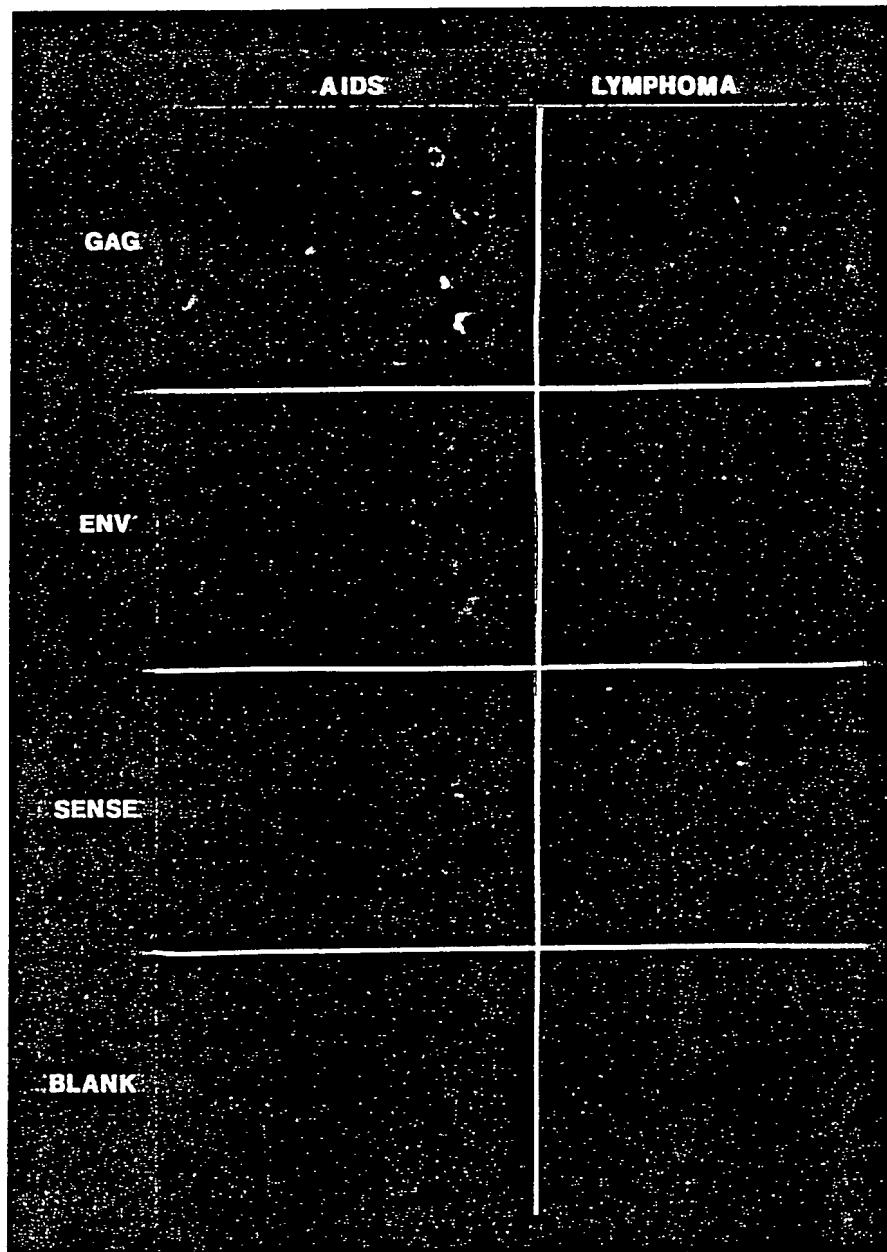


FIG. 9

SUBSTITUTE SHEET

10 | 20

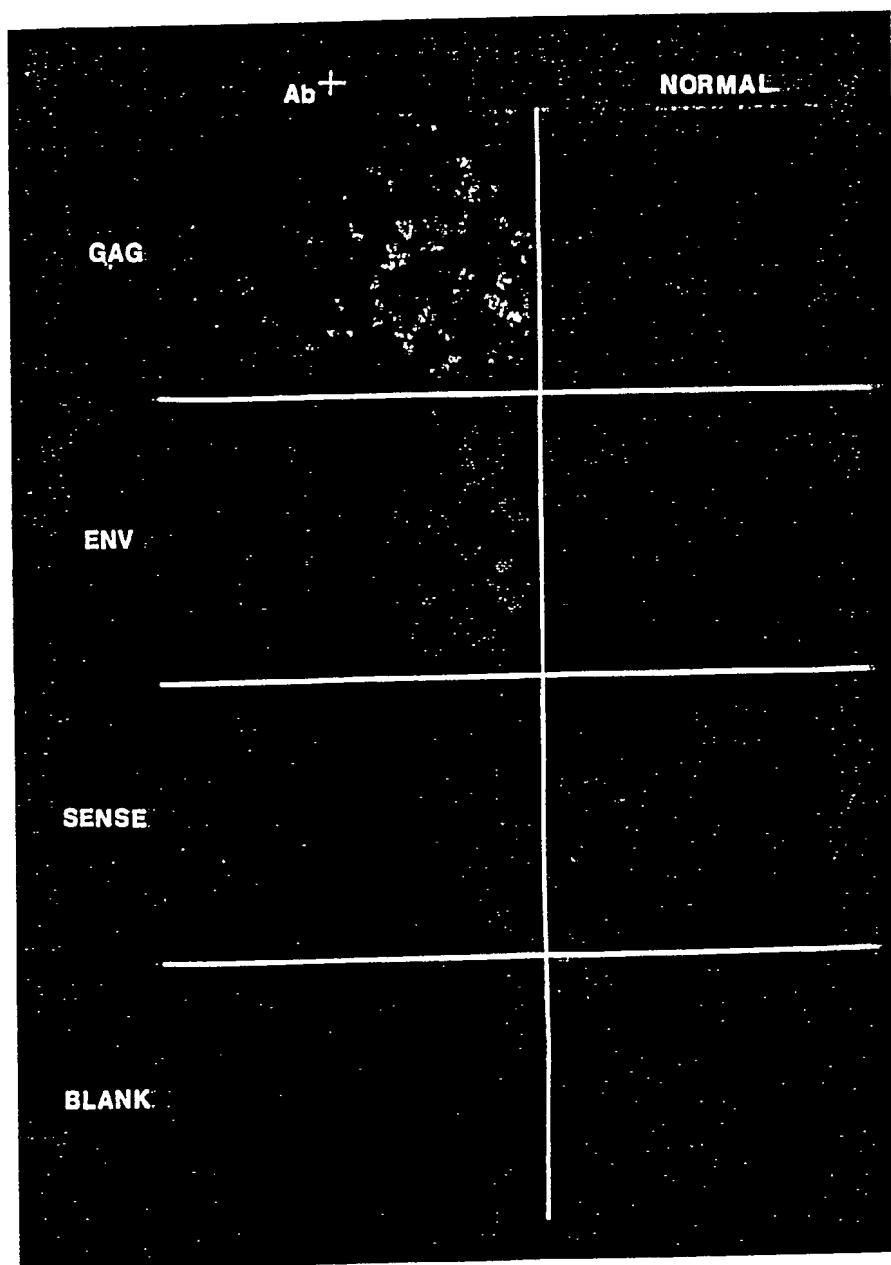


FIG. 10

SUBSTITUTE SHEET

11/20

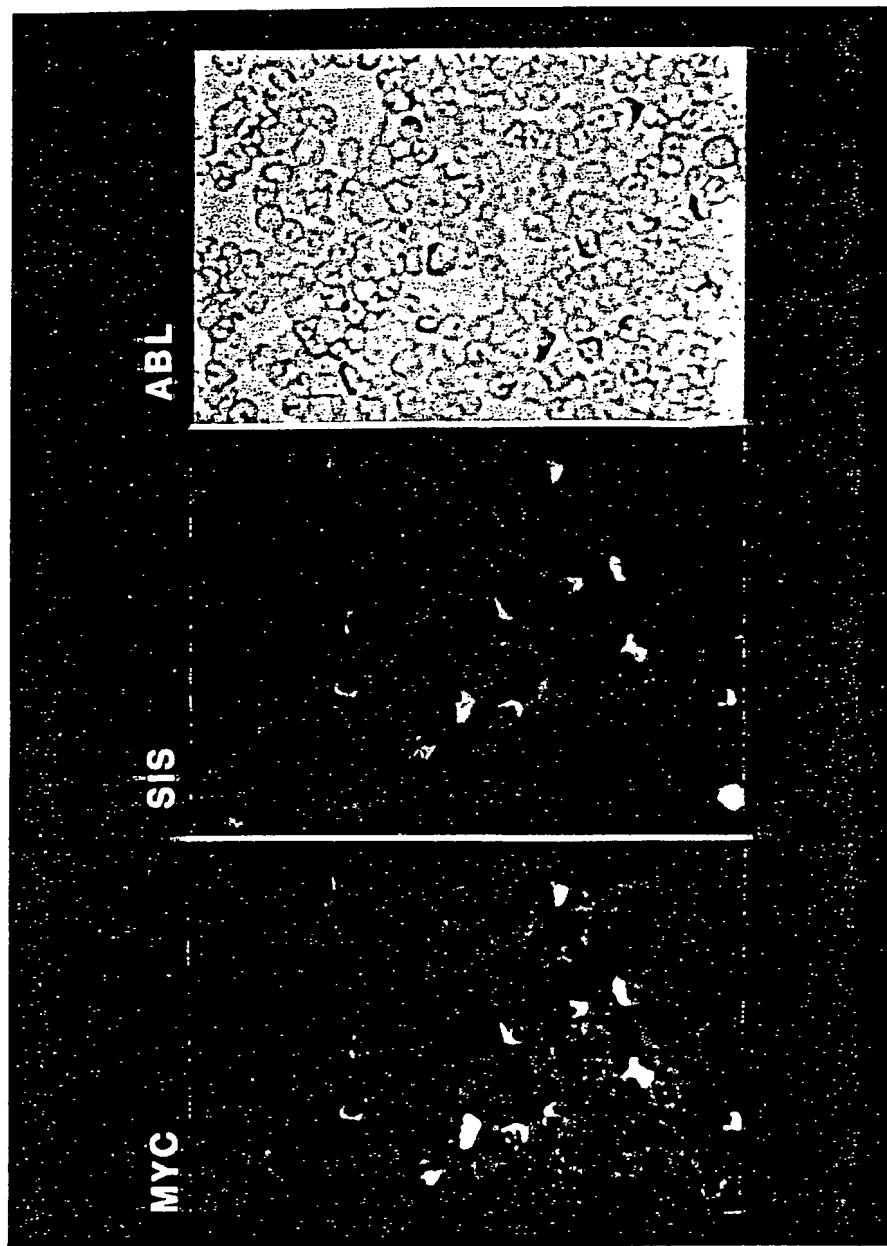


FIG. 11

SUBSTITUTE SHEET

12/20

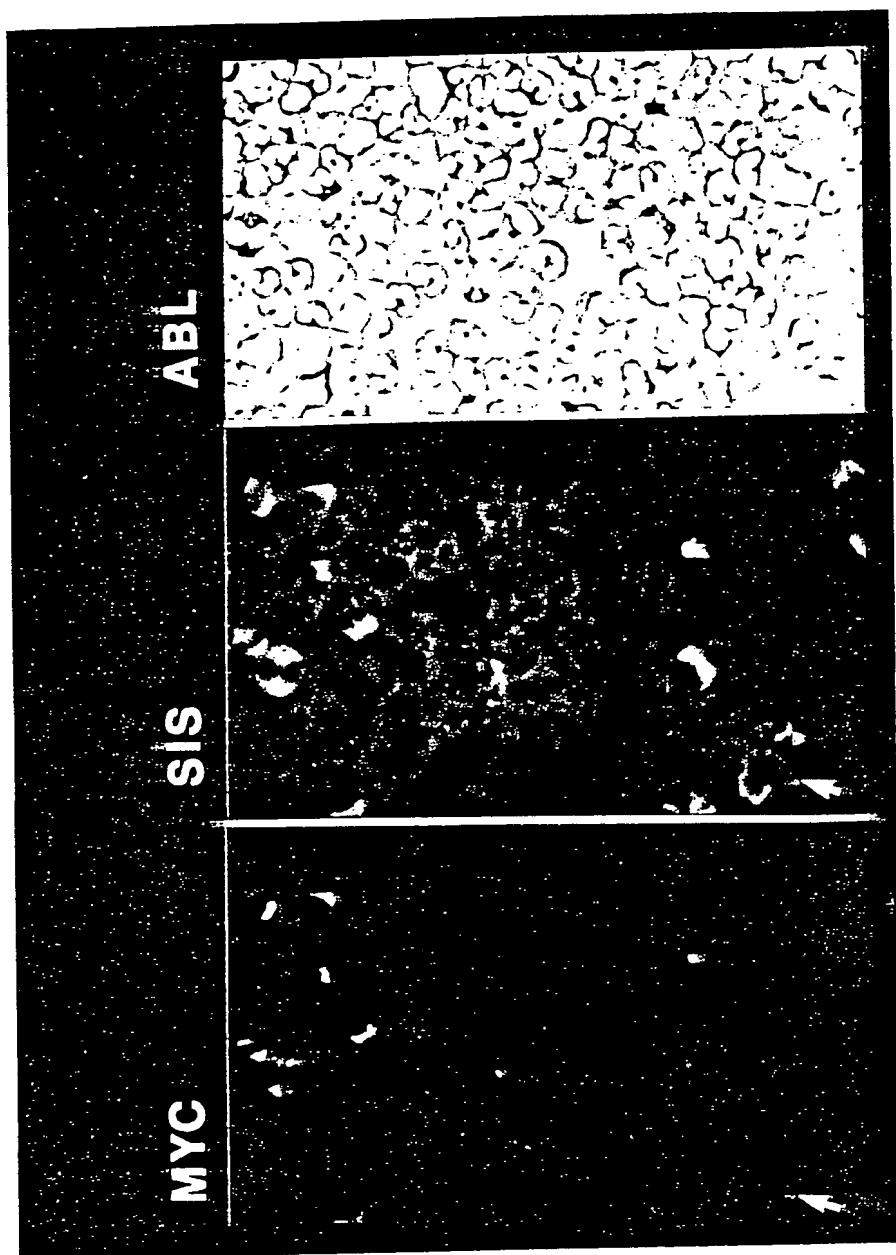


FIG. 12

SUBSTITUTE SHEET

13/20

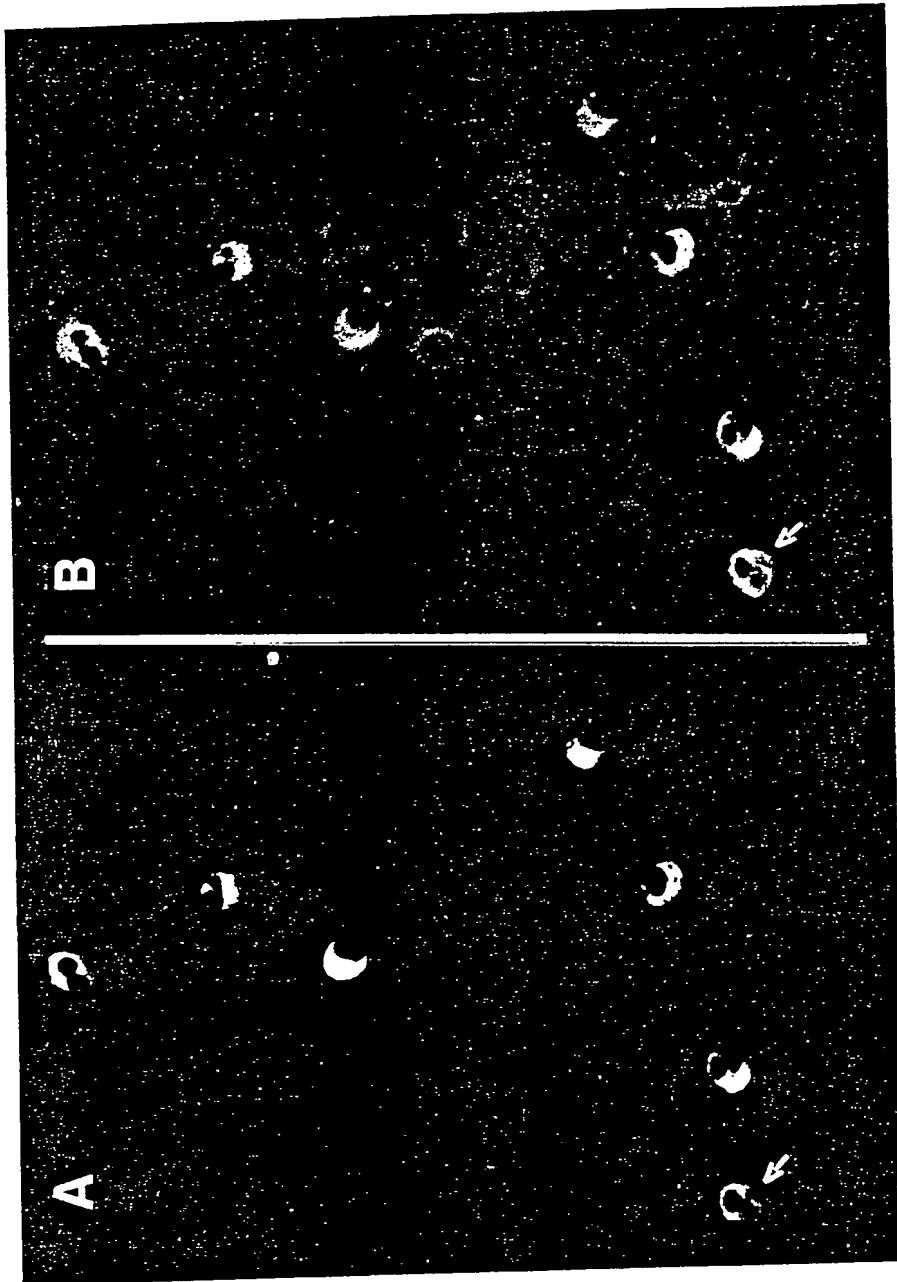


FIG. 13

SUBSTITUTE SHEET

14 | 20

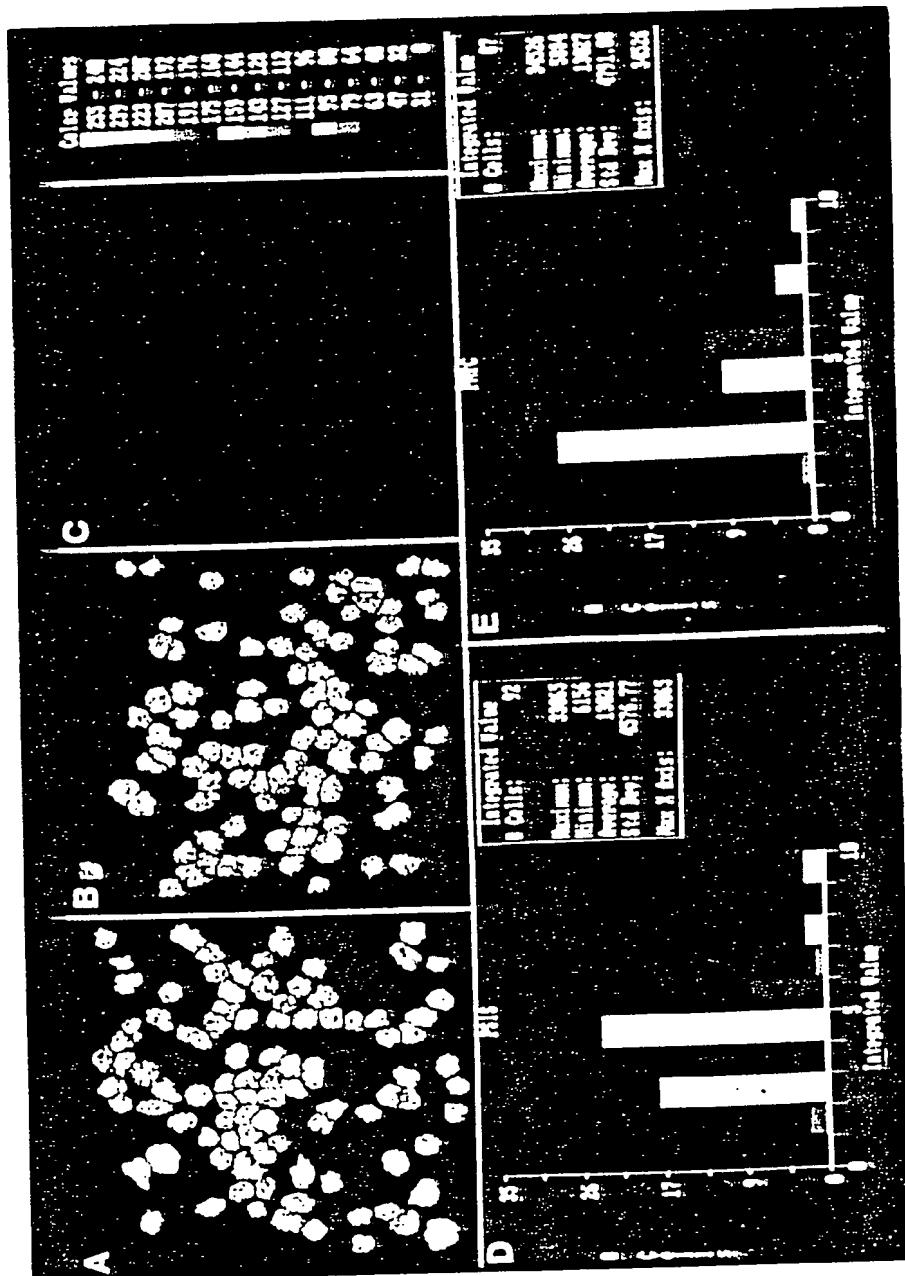


FIG. 14

SUBSTITUTE SHEET

15/20

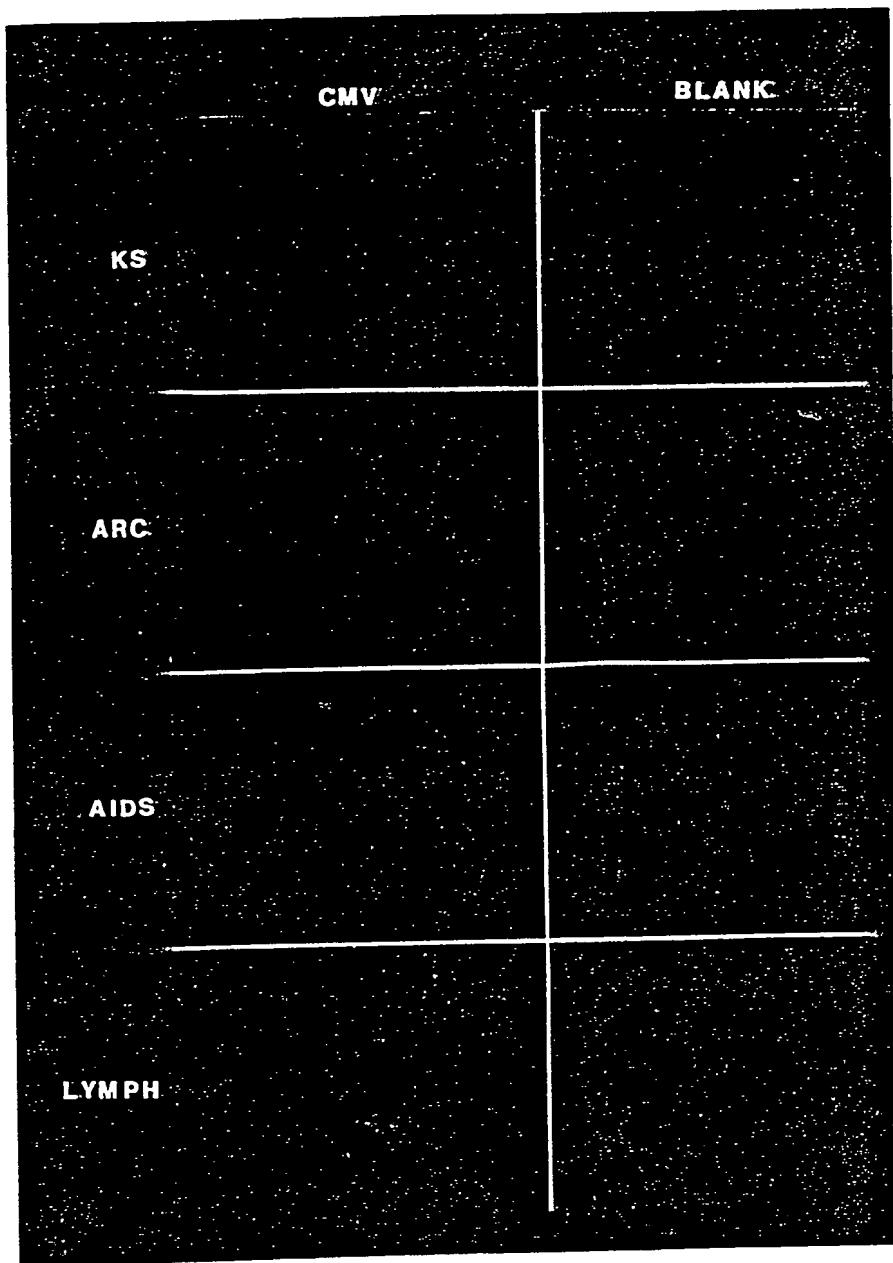


FIG. 15

SUBSTITUTE SHEET

16/20

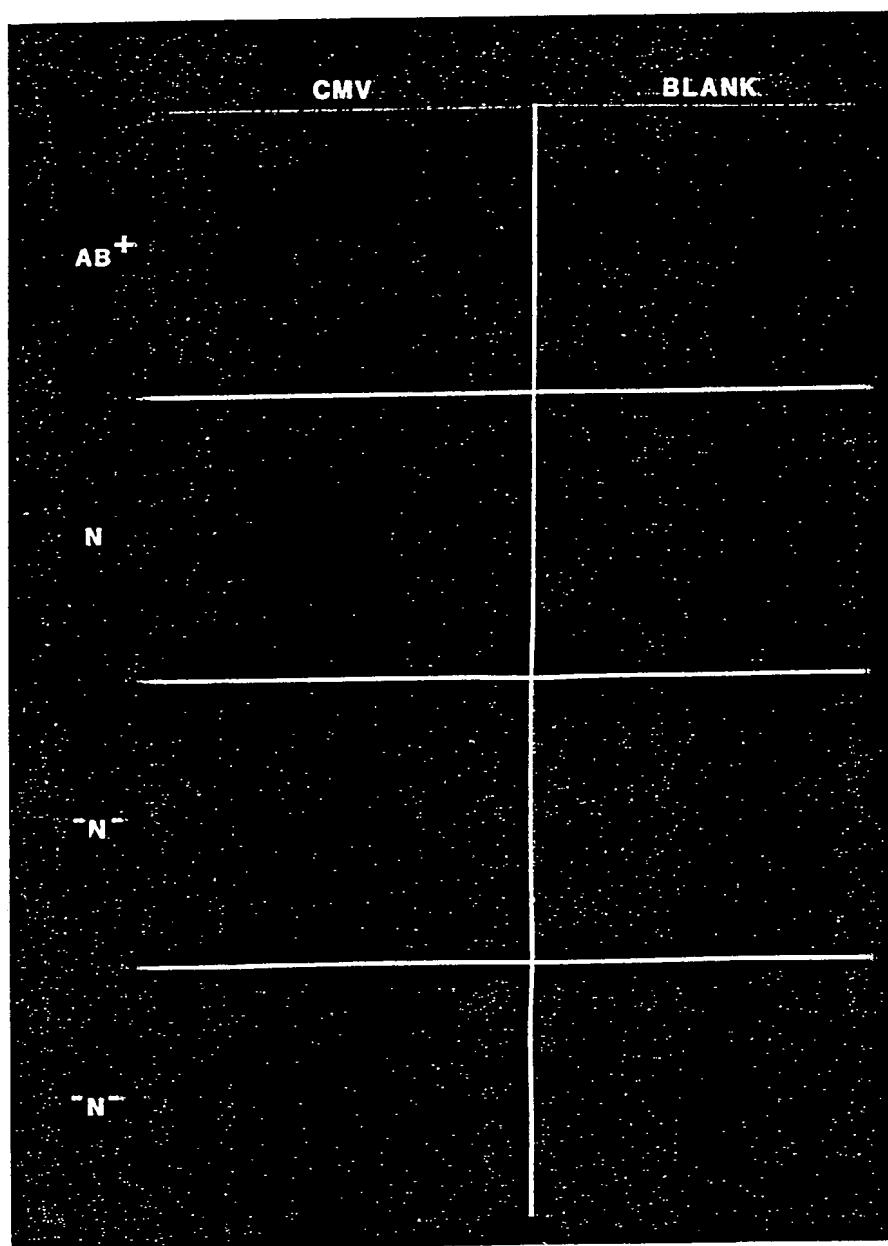


FIG. 16

SUBSTITUTE SHEET

17/20

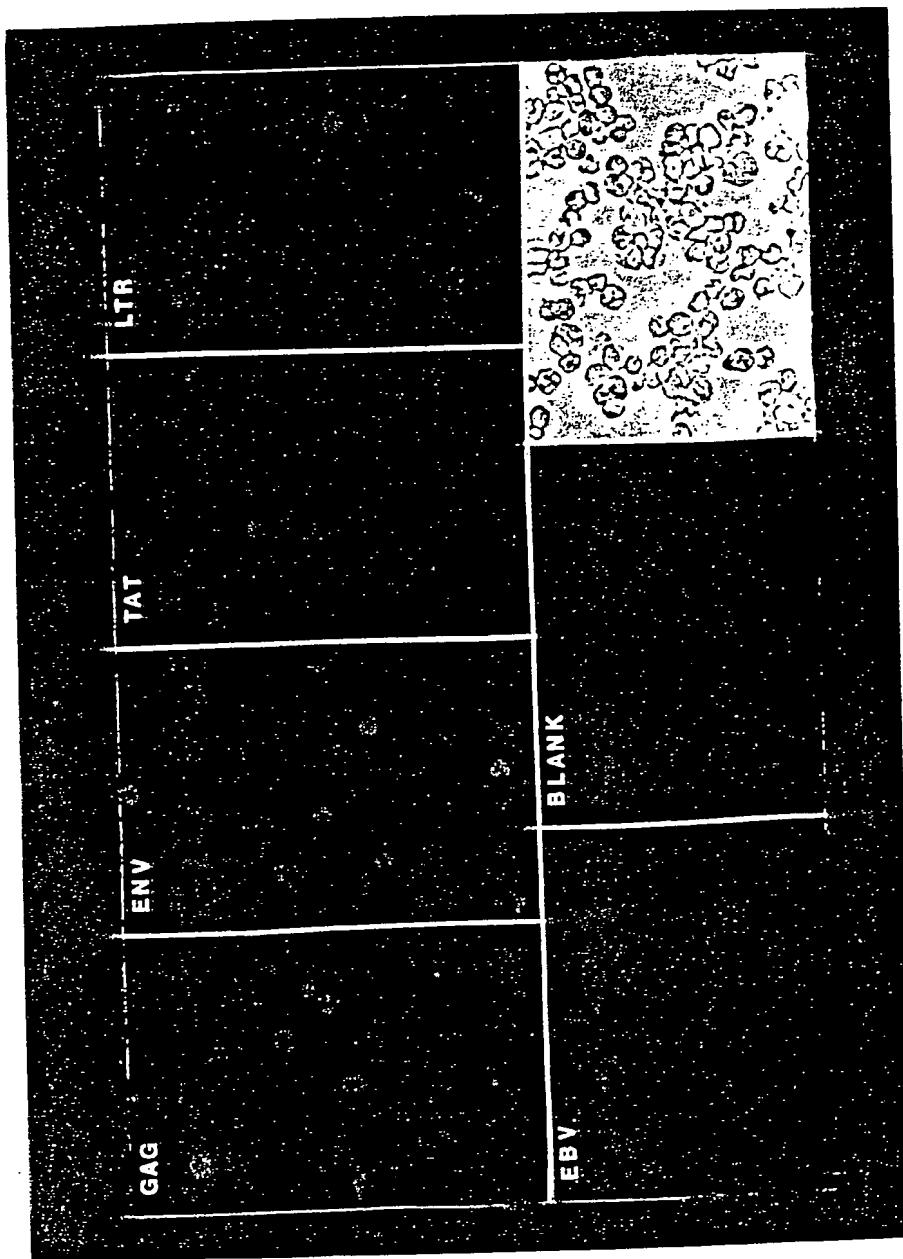


FIG. 17

SUBSTITUTE SHEET

18/20

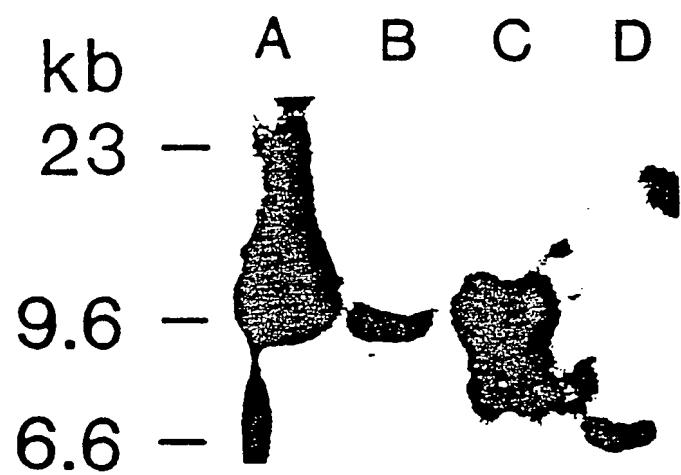


FIGURE 18

19/20

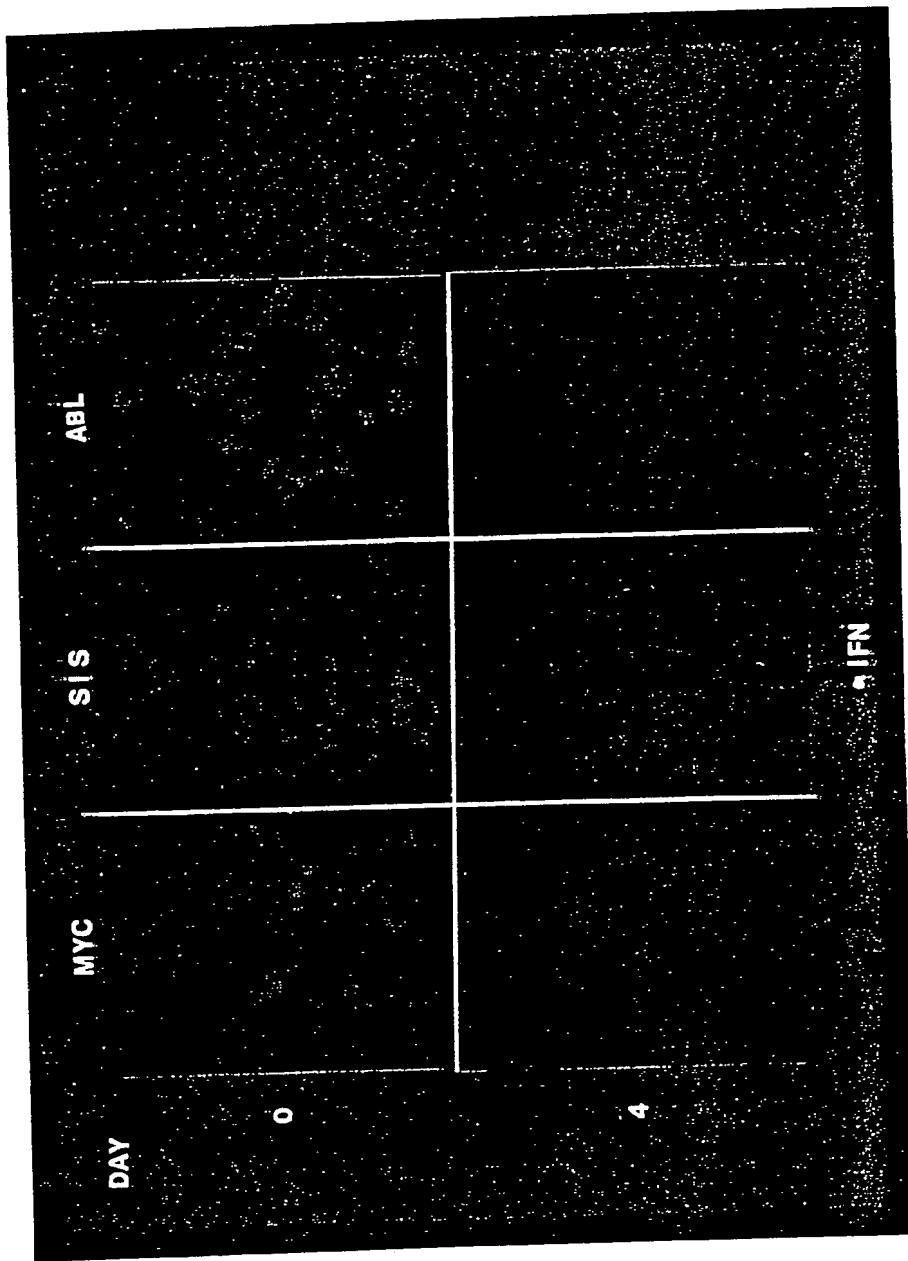


FIG. 19

SUBSTITUTE SHEET

20|20

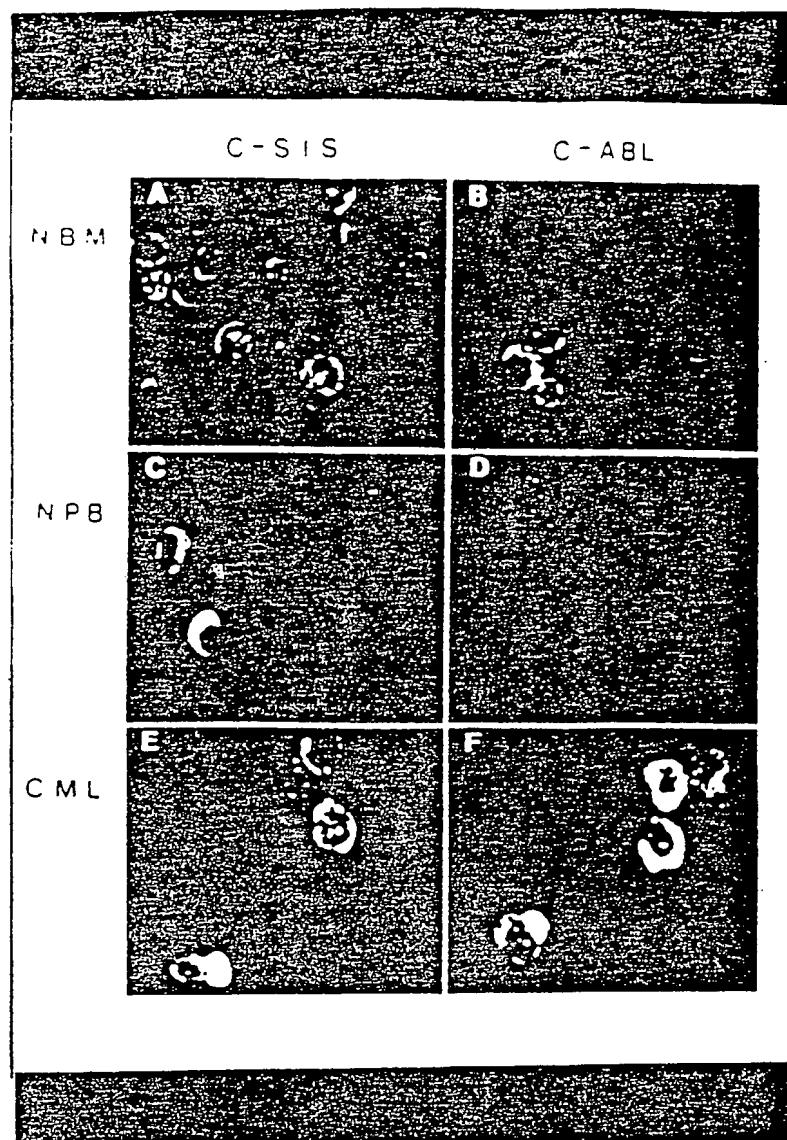


FIGURE 20

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03582

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC
 IPC(4): C12Q 1/68; G01N 33/53
 U.S. Cl.: 435/6, 7

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷	
Classification System	Classification Symbols
U.S.	435/6, 7
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸	

Computer Search: Cas, Biosis; APS Data bases

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	WO, A, 86/04146 (United States of America) 17 July 1986.	1-23
Y	WO, A, 85/04720 (Howard Florey Institute of experimental Physiology and Medicine) 24 October 1985	1-23
Y	Laboratory Investigation, Volume 56 (1987) page 88A Wolber: "Cytomegalovirus Detection by <u>In-situ</u> , DNA Hybridization"	1-23
Y	Chemical Abstracts, Volume 107 number 19 issued 1987 (Columbus, Ohio USA) Bresser et al. "Comparison and optimization of <u>in situ</u> hybridization procedure yielding rapid, sensitive mRNA detections" abstract No. 171731w, Gene Anal Tech 4(5) 89-104 1987	1-23
		1-23

* Special categories of cited documents: ¹⁰

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

01 November 1989

Date of Mailing of this International Search Report

08 DEC 1989

International Searching Authority

ISA/US

Signature of Authorized Officer


Scott A. Chambers

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Journal of Clinical Microbiology, Vol 22 No. 4 issued October 1985 (Washington D. C. USA) Forghani et al. "Rapid detection of Herpes Simplex Virus DNA in human Brain Tissue by <u>In situ</u> Hybridization	1-23
---	--	------

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers _____, because they relate to subject matter^{1,2} not required to be searched by this Authority, namely:

2. Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out^{1,2}, specifically:

3. Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.